Conformational changes within the HLA-A1:MAGE-A1 complex induced by binding of a recombinant antibody fragment with TCR-like specificity

Pravin Kumar,^{1†} Ardeschir Vahedi-Faridi,^{2†} Wolfram Saenger,² Andreas Ziegler,^{1*} and Barbara Uchanska-Ziegler¹

¹Institut für Immungenetik, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Freie Universität Berlin, Thielallee 73, Berlin 14195, Germany

²Institut für Chemie und Biochemie/Kristallographie, Freie Universität Berlin, Takustrasse 6, Berlin 14195, Germany

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Abstract: Although there is X-ray crystallographic evidence that the interaction between major histocompatibility complex (MHC, in humans HLA) class I molecules and T cell receptors (TCR) or killer cell Ig-like receptors (KIR) may be accompanied by considerable changes in the conformation of selected residues or even entire loops within TCR or KIR, conformational changes between receptor-bound and -unbound MHC class I molecules of comparable magnitude have not been observed so far. We have previously determined the structure of the MHC class I molecule HLA-A1 bound to a melanoma antigen-encoding gene (MAGE)-A1-derived peptide in complex with a recombinant antibody fragment with TCR-like specificity, Fab-Hyb3. Here, we compare the X-ray structure of HLA-A1:MAGE-A1 with that complexed with Fab-Hyb3 to gain insight into structural changes of the MHC molecule that might be induced by the interaction with the antibody fragment. Apart from the expulsion of several water molecules from the interface, Fab-Hyb3 binding results in major rearrangements (up to 5.5 Å) of heavy chain residues Arg65, Gln72, Arg145, and Lys146. Residue 65 is frequently and residues 72 and 146 are occasionally involved in TCR binding-induced conformational changes, as revealed by a comparison with MHC class I structures in TCR-liganded and -unliganded forms. On the other hand, residue 145 is subject to a reorientation following engagement of HLA-Cw4 and KIR2DL1. Therefore, conformational changes within the HLA-A1:MAGE-A1:Fab-Hyb3 complex include MHC residues that are also involved in reorientations in complexes with natural ligands, pointing to their central importance for the peptide-dependent recognition of MHC molecules.

Keywords: HLA-A1; X-ray crystallography; peptide-restricted antibody; T cell receptor; ligand-induced conformational changes

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[†]Pravin Kumar and Ardeschir Vahedi-Faridi contributed equally to this work.

*Correspondence to: Andreas Ziegler, Institut für Immungenetik, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Freie Universität Berlin, Thielallee 73, 14195 Berlin, Germany. E-mail: andreas.ziegler@charite.de

Introduction

Major histocompatibility complex (MHC, in humans HLA) class I molecules are heterotrimeric complexes consisting of a heavy chain (HC), a noncovalently associated light chain, β_2 -microglobulin (β_2m), and a peptide. These heterotrimers are also referred to as peptide:MHC complexes (pMHC). Within the endoplasmic reticulum, HC: β_2m complexes specifically bind small fragments of degraded proteins, are then

transported as intact heterotrimers to the surface of antigen presenting cells and subsequently serve as targets for receptors on effector cells such as T or natural killer (NK) cells.¹ Although typical affinities of individual interactions are low, in the micromolar range, the recognition process is highly specific. It permits T cell receptors (TCR) on T cells to distinguish peptides derived from self-antigens and foreign, for example, viral, molecules.² In contrast, the binding of killer cell Ig-like receptors (KIR) on NK cells is more promiscuous, because only residues in the vicinity of the peptide C-terminus of a pMHC are involved in the interaction.^{3,4} However, the general principles are not yet fully understood that govern recognition or lack of interaction between pMHC and TCR or KIR and determine the biological outcome of an encounter between an effector cell and its target. Particularly puzzling is the occasionally drastic influence of subtle conformational changes of residues within a pMHC complex or a TCR on the response of T cells.^{2,5–7}

The polymorphic nature of MHC class I antigens enables them to present a diverse range of peptides, generally varying between 8 and 12 residues in length. These peptides are anchored in the peptide binding groove that is formed by HC residues through specific interactions between side chains of the peptide and residues shaping the peptide-binding pockets of an MHC molecule.^{1,8} The specificity of peptide presentation is usually determined by only one or two of such pockets that accommodate primary anchor residues of a peptide,⁹ with one or more secondary anchors that fine tune the binding motifs for a given MHC class I antigen.¹⁰ For example, HLA-A*010101 is a human MHC class I allele whose product preferentially binds nonapeptides with the anchor residues Asp or Glu at position 3 and Tyr at position 9. Thr or Ser at position 2 and Leu at position 7 are auxiliary anchor residues.^{11–13}

We have previously determined the structure of a recombinant antibody fragment, Fab-Hyb3 (Hyb3 in short), that reacts with HLA-A1 in a peptide-dependent fashion.¹⁴ Within this complex, HLA-A1 is bound to the peptide MAGE-A1 (EADPTGHSY), which is derived from the melanoma antigen-encoding gene (MAGE)-A1 molecule (residues 161–169). To some extent, the binding of the *in vitro* affinity-matured Hyb3 resembles that of a soluble TCR or KIR, but Hyb3 exhibits a ~1000 times higher affinity toward its target than a typical TCR or KIR.^{2–4,15} In attempting to understand the basis for this finding, we focus here on changes induced by Hyb3 in the conformation of pMHC residues by comparing the Hyb3-liganded structure (A1:MAGE-A1:Fab-Hyb3, AMF) with that of the newly determined, unliganded HLA-A1:MAGE-A1 complex (AM). The results reveal that four residues, at positions 65, 72, 145, and 146 of the HC, undergo highly significant changes in their side chain orientations because of the interaction with Hyb3. All of these

residues are also involved in TCR or KIR binding-induced conformational changes in selected other pMHC.

Results

Structural features of the HLA-A1:MAGE-A1 complex

The HLA-A1 (AM) complex crystallized in space group P2₁2₁2₁ (Table I) with one molecule of the complex in the asymmetric unit. The structure was determined at 1.8 Å resolution and refinement converged at R_{cryst} of 0.194. The electron density map for the structure is of high quality, as shown for the MAGE-A1 peptide [Fig. 1(A)]. Several nonspecific contacts between HLA-A1 complexes stabilize the crystal lattice. However, the bound MAGE-A1 peptide does not participate in these crystal contacts, thereby permitting a detailed comparison with the peptide in the AMF complex. The overall structure of HLA-A1 exhibits the typical HLA class I topography.¹

The MAGE-A1 peptide was modeled unambiguously into the electron density, revealing relatively inflexible side chain orientations of peptide residues as seen in the qualitative B factor plot [Fig. 1(B)], with the exception of pHis⁷. Apart from this residue and residues pPro⁴, pThr⁵, and pGly⁶, which are slightly more flexible in HLA-A1, as well as pGlu¹, which exhibits higher flexibility in the AMF [Fig. 1(C)] than in the AM complex [Fig. 1(B)], the B factor values of peptide residues are very similar in both structures. The MAGE-A1 peptide is buried in the peptide binding groove [Fig. 1(D,E)] and provides only a relatively flat surface for recognition by a TCR or Hyb3. A large number of polar and nonpolar interactions between the MAGE-A1 peptide and HC residues that line the peptide binding groove as well as water-mediated intrapeptide hydrogen bonds stabilize this conformation (not shown). The peptide residues pGlu¹, pAla², pAsp³, pHis⁷, and pPhe⁹ are accommodated, respectively, in the A, B, D, E, and F pockets of the peptide binding groove [Fig. 1(E)]. The solvent accessibility of the MAGE-A1 peptide residues demonstrates varying degrees of solvent exposure for peptide residues, with pPro⁴, pThr⁵, pGly⁶, and pSer⁸ being the most “exposed” residues (solvent accessible surface area (SASA) values per residue: 89.6, 39.7, 43.5, and 52.4 Å², respectively) and pAla², as well as the anchor residues pAsp³ and pTyr⁹ being the most “occluded” residues (SASA values per residue: 3.9, 10.2, and 7.0 Å², respectively).

3D comparison of the AM and the AMF structures

A comparison of HLA-A1 with the liganded complex¹⁴ reveals a number of differences, although the overall structures are very similar. The root mean square (rms) deviation values obtained upon Cα overlay of

Table I. Crystal Data Collection and Refinement Statistics for the HLA-A1:MAGE-A1 Complex

Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Unit cell	
<i>a</i> (Å), <i>b</i> (Å), <i>c</i> (Å)	51.176, 74.060, 125.940
Resolution (Å)	63.89–1.80 (1.84–1.80)
Unique reflections	41526 (2954)
Redundancy	4.0 (4.2)
Completeness (%)	96.78 (94.75)
<i>I</i> /σ	17.3 (3.82)
<i>R</i> _{sym} ^a	0.071 (0.337)
Refinement	
Resolution (Å)	63.89–1.80 (1.84–1.80)
Reflections	41526 (2954)
<i>R</i> _{cryst} ^b	0.194 (0.280)
<i>R</i> _{free} ^c	0.224 (0.337)
HLA-A1 heavy chain, no. of atoms/average B value (Å ²) (chain A)	2241/16.73
β ₂ -microglobulin, no. of atoms/average B value (Å ²) (chain B)	853/17.39
MAGE-A1 peptide, no. of atoms/average B value (Å ²) (chain C)	69/19.35
Water, no. of molecules/average B value (Å ²)	587/30.44
Glycerol, no. of molecules/average B value (Å ²)	3/35.53
Estimated overall coordinate error (Å)	0.136
Root mean square deviation from ideal geometry	
Bond length (Å)	0.015
Bond angles (°)	1.451
Ramachandran plot	
Most favored regions (%)	92.8
Additionally allowed regions (%)	6.9
Generously allowed regions (%)	0.3
Disallowed regions (%)	0.0

Values for highest resolution shell are indicated in parentheses.

^a $R_{\text{sym}} = \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_i I_{h,i}$.

^b $R_{\text{cryst}} = \sum_h |F_o - F_c| / \sum F_o$ (working set, no σ cutoff applied).

^c R_{free} is calculated the same way as R_{cryst} , but for 5% of the data excluded from refinement.

the two complexes are 0.56 Å (for the entire complex), 0.58 Å (only HC), 0.30 Å (only β₂m), and 0.37 Å (only peptide). Independent superposition of individual domains of the HLA-A1 HC shows that the α3 domain exhibits slightly more differences (Cα rms deviation of 0.40 Å) when compared with the α1/α2 domains (Cα rms deviation of 0.38 Å). To observe main chain differences in the peptide binding groove, we overlaid the main chain atoms of the α1- and α2-domains, including all peptide binding groove residues (1–180) of the AMF complex over the corresponding residues of the AM structure (see Fig. 2). This analysis reveals that, for certain locations, particularly in loop regions such as residues 39–42 of the α1-domain and a conspicuous stretch of three residues (149–151) within the N-terminal region of the α2-helix, the positions of the Cα atoms in the two structures differ by more than 1.0 Å. Similar domain-wise analyses of other regions of the structure show Cα shifts larger than 0.5 Å at several positions in the α3 domain and at residues 46–49, 59, and 96–99 in β₂m. The comparison demonstrates also that the α3-domain has most main chain shifts (0.50–1.13 Å) and differences in side chain orientations, although nearly all residues having such differences are part of loop regions.

By clicking on any part of the conventional 2D Figure 3 in the PDF, access to the 3D feature is

obtained through the freely available Adobe Reader (Version 9) on any Windows-based computer with a modern graphic card (128+ MB memory) and sufficient RAM (1024+ MB). The “Help” option within the program provides an introduction into the possibilities that are offered. Zoom, rotation in freely chosen directions, or hiding of structural elements (β₂m, HC, peptide, each in one or both of the two structures) permit an interactive access. A better understanding of many structural features is thus provided. In the present case, it is particularly helpful to compare the peptide conformations by zooming in and by rotating the two structures with the HC component hidden (by toggling its visibility off in the model tree) or by comparing the conformation of the HC residues Arg65, Gln72, Arg145, and Lys146, all of which are contacted by Hyb3 (see later).

The preset views permit the reader to follow a “tour” of the molecule resembling a prerendered video file of the structures. However, other than a film, the tour presented here still permits full interaction with the structures at any stage. It begins by depicting the entire HLA-A1 complex from different angles (views 1–3 of the model tree). It then shows a top view of the α1- and α2-domains that form the peptide binding groove with the peptide (view 4) followed by a side view with the peptide, still partially hidden behind the

Figure 1. Orientation of the MAGE-A1 peptide in the HLA-A1:MAGE-A1 complex. (A) $2F_o - F_c$ electron density contoured to 1.5σ level around the MAGE-A1 peptide in the HLA-A1 structure. (B, C) B-factor plot of the MAGE-A1 peptide in the AM (B) and in the AMF complexes (C). Only the imidazole ring of pHis⁷ is considerably more flexible in the unliganded structure. (D, E) Orientation of the peptide in the peptide binding groove of HLA-A1 in cartoon (D, $\alpha 2$ -helix removed for clarity) and surface representations (E). pGlu¹, pAla², pAsp³, pHis⁷, and pPhe⁹ are accommodated, respectively, in the A, B, D, E, and F binding pockets. In all images, the peptide N-terminus is on the left and the C-terminus on the right.

$\alpha 2$ -helix (view 5), or freely visible with the $\alpha 2$ -helix removed (view 6), and then concentrates on four HC residues that undergo conformational changes upon Hyb3 engagement (views 7, 8). The next series of pictures show initially a view of the entire AMF complex with the six complementarity determining regions (CDR) of Hyb3 (views 9, 10), then zoom into the contact area (view 11), depict the conformations of the four above-mentioned HC residues in liganded as well as unliganded form together with the $\alpha 1$ - and $\alpha 2$ domains of the two structures superimposed (views 12, 13), and finally concentrate on the MAGE-A1 peptide that exhibits two slightly distinct conformations when AM and AMF are compared (views 14–16), before the AM complex is shown again (views 17, 18).

Anchoring of MAGE-A1 within the peptide binding groove of AM and AMF

The MAGE-A1 peptide adopts nearly identical conformations in the peptide binding grooves of the Hyb3-liganded and -unliganded structures (Figs. 1 and 3). The middle part of the peptide is buried slightly deeper in the groove in the unliganded form compared with the liganded structure, as C_α shifts of 0.5, 1.1, and 0.6 $\text{\AA}$ are observed for pThr⁵, pGly⁶, and pHis⁷, respectively (see views 14, 16 of the “tour” provided as part of the interactive 3D representation in Fig. 3). This result is different from that described for the A6 TCR and HLA-A2:Tax,¹⁶ where TCR binding results in pushing the peptide further into the binding groove (2.7 $\text{\AA}$ for the C_α atom of pPro⁶; 4.6 $\text{\AA}$ for C_γ of

Figure 2. Comparison of HLA-A1 HC in unliganded and liganded forms. A main-chain overlay of the α 1- and α 2-domains of the HLA-A1 HC in unliganded and Hyb3-liganded forms is shown. Residues with C α shifts larger than 1 Å are colored in green (Hyb3-liganded) and orange (unliganded). The most remarkable shifts are observed in the region of the peptide binding groove near the C-terminus of the MAGE-A1 peptide (on the right of the image).

pVal⁷). A comparison of the solvent accessibility of the peptide residues reveals that pGly⁶ and pSer⁸ have 18% and 20% higher accessibility, respectively, in HLA-A1, while the values for the other peptide residues are very similar (results not shown).

Two intrapeptide water-bridged hydrogen bonds are present in both structures. The N and O atoms of pAla² form a hydrogen bond with a water molecule (WC217 and WX1, respectively), whereas pPro⁴O and pGly⁶N contact water molecules at comparable locations in the AM and AMF complexes (not shown). On the other hand, a further water-mediated intrapeptide hydrogen-bond between the same peptide backbone atoms (via WX4) is present only in the Hyb3-liganded structure [compare Fig. 4(A,B)]. It is likely that this feature facilitates the interaction of pPro⁴ and pGly⁶ with the Trp52A residue of the Hyb3 heavy chain, which is mediated by the same water molecule.¹⁴ However, both structures also exhibit hydrogen bonding features that are not found in the other (see Fig. 4). In the AM complex, pGlu¹ contacts Arg163 indirectly via water WA631, while a comparable water-mediated contact is missing in the liganded complex. In contrast, direct hydrogen bonds linking pGlu¹ with Arg163 or pSer⁸ with Trp147 are largely retained in both structures with minor differences in bond lengths. This is exemplified by Arg114 and Arg156: although distinct in detail, the side chains of these residues are coordinated similarly, via a water molecule, to pHis⁷ (AM complex) or to pThr⁵ and pHis⁷ (AMF complex).

Finally, pSer⁸ is contacted by Trp147 only in the former structure [Fig. 4(A)].

Ligand-induced reorientations of HLA-A1 HC residues

Hyb3 binding is associated with significant side chain orientational perturbations (>2.0 Å) of four HC residues, Arg65, Gln72, Arg145, and Lys146 that show also high buried SASA (BSASA), with Arg65 and Gln72 being the only residues exhibiting values larger than 80 Å².¹⁴ In the AM complex, Arg65 of the HC forms water (WA508)-mediated hydrogen bonds with two oxygens (Glu58O and Glu58OE1) and a direct hydrogen bond with Gln62. Furthermore, the side chain of Gln72 is aligned along the α 1 helix, contacting Arg75 both directly and indirectly through WA574 in this structure [Fig. 5(A)]. In contrast, in the AMF complex, water molecules are expelled from this area of the interface between the two molecules. Although the diverging resolutions of the two structures do not allow accurate estimates to be made, there are 94 water molecules in the vicinity of the α 1- and α 2-domains in the AM structure that are not present in the AMF complex (results not shown). As a consequence, Arg65NH1,2 contact Asp30 and Asp31 within CDR-1 of the Hyb3 heavy chain (CDR-H1) through three direct hydrogen bonds, whereas Gln72 interacts with the residues Tyr100C and Phe98 within CDR-H3 [Fig. 5(B)]. Furthermore, the Arg65CZ atom is shifted by 2.73 Å between the two structures, whereas the position of the Gln72CD atom differs by 2.32 Å [Fig. 5(A,B)]. In addition, the contact of Gln72O with Arg75N is shorter in the unliganded structure (3.31 Å as opposed to 3.60 Å).

Further substantial reorientations can be observed for residues that are part of the N-terminal portion of the α 2-helix [Fig. 5(C,D)]. In HLA-A1, the side chain nitrogen atoms of Arg145 form a network of water-mediated contacts with Gln141 and Met138 [Fig. 5(C)]. Arg145 engages also in a hydrogen bond with a symmetry-related molecule (not shown). Lys146NZ forms indirect, water-mediated contacts with pTyr⁹, pSer⁸, and Tyr84 [Fig. 5(C)]. Again, water molecules do not participate any more in the interactions of Arg145 and Lys146 in the AMF complex [Fig. 5(D)]. Arg145 does not engage in any direct or indirect interactions with other HC residues, but instead contacts Arg94 of Hyb3 within CDR-3 of the light chain (CDR-L3) [Fig. 5(D)]. On the other hand, Lys146 forms direct hydrogen bonds with the CDR-L3 residues Ser93 and Asp95A, as well as with the HLA-A1 HC residue Tyr84 and with the C-terminal peptide residue pTyr⁹ [Fig. 5(D)]. In addition, Lys146 contacts Asp95A of CDR-L3 also by van der Waals interactions.¹⁴ The position of the Arg145CZ atom differs by 5.54 Å and that of the Lys146NZ atom by 3.60 Å between the AM and AMF complex [Fig. 5(C,D)].

Figure 3. An interactive 3D model depicting conformational changes of HLA-A1:MAGE-A1 in unliganded and liganded forms. The HLA-A1 HC is shown in light gray, β_2m in dark gray, the H-chain and L-chain of Hyb3 also in light gray. The MAGE-A1 peptide is in orange (HLA-A1) and green (AMF), and the complementarity determining regions of Hyb3 are colored blue (CDR-H1), green (CDR-H2), sky-blue (CDR-H3), orange (CDR-L1), red (CDR-L2), and yellow (CDR-L3), respectively. **(A)** View into the binding groove of the HLA-A1 molecule, with the peptide in place. **(B)** Overlay of the peptide binding groove and the MAGE-A1 peptide as displayed in the unliganded and the Hyb3-liganded HLA-A1 molecule. **(C)** The HLA-A1:MAGE-A1:Hyb3 complex seen from the side of the $\alpha 2$ -helix of the HLA-A1 molecule. 3D functions are available through a click on any part of the image in the PDF version of the article (the 3D features can be terminated by right-clicking on the 3D display and selecting the “Disable 3D” function from the contextual menu). The model tree icon permits to access individual components of the 3D model. Note that ligHLA-A1 is abbreviated as “AMF” (A1:MAGE-A1:Fab-Hyb3) in the view designations provided as part of the model tree. The displays which the tour presents are briefly designated at the left. Each model can be interactively manipulated using the mouse (rotating, panning and zooming tools can be selected through the toolbar or contextual menu), and readers can access the preset “tour” of the model by clicking on the respective designations (either via the middle section of the model tree when it is displayed, or the drop down menu in the toolbar, or through the contextual menu). By rotating the molecule and by zooming into regions of interest, the understanding of many molecular features is facilitated, in particular the conformational changes of HC residues. Initially, the tour shows views of HLA-A1 from the side, along the binding groove, from the top, and finally depicts the HC residues that undergo conformational changes after being contacted by Hyb3. Thereafter, the Hyb3-liganded complex is considered, and several views are offered that eventually zoom into the contact area between HLA-A1 and Hyb3, demonstrating the conformational changes.

Most of the HC residues that contact the peptide via hydrogen bonds in the AM structure have similar BSASA values also in the AMF complex (not shown). However, there are a number of exceptions: Asn66, Ala69, Thr73, Tyr84, Lys146, and Gln 155 show significant differences in BSASA values due to peptide contacts in the two structures. The BSASA values diverge particularly strongly for Thr73 and Lys146. Each of these two residues contributes $\sim 40 \text{ \AA}^2$ of the total

HLA-A1 HC surface area that is buried due to peptide binding in HLA-A1, but these values are more than 50% lower in the AMF complex. Gln155, one of the five residues that exhibits $>50 \text{ \AA}^2$ BSASA in the AM complex, shows a 32.6% reduced BSASA in the AMF structure. Finally, Ala69 and Tyr84, although only small contributors to BSASA (10.2 and $8.3 \text{ \AA}^2$, respectively), reveal BSASA values that are about 80% lower in the Hyb3-liganded structure. Overall, the reduction

Figure 4. Water-bridged hydrogen bonds involving the MAGE-A1 peptide in unliganded and Hyb3-ligated structures. The HLA-A1 HC is shown in shades of gray in both figures. Ordered water molecules mediating hydrogen bonds are shown in red. Water molecules are labeled with a W succeeded by their chain id in the respective PDB files followed by their number. **(A)** HLA-A1 molecule. The MAGE-A1 peptide is shown in orange and HLA-A1 HC in gray. The pGlu¹OE1 and OE2 atoms form a hydrogen bond with Arg163NH₂ through WA631, the pHis⁷N and O atoms contact Arg114NH₂ and Arg156NH₂ through WC419, and the Trp147NE1 atom forms hydrogen bonds with pSer⁸N and OG atoms through WC267. **(B)** AMF complex. The MAGE-A1 peptide is shown in green, HLA-A1 HC in white and part of the Hyb3-H chain in yellow. pPro⁴O and pGly⁶N interact with Trp52ANE1 of the heavy chain of Hyb3 through WX4, whereas Arg156NH₂ and Arg114NH₂ contact pThr⁵O and pHis⁷N by hydrogen bonds through WA345.

in BSASA between the unliganded and the liganded structures is about 16% when HC residues involved in hydrogen-mediated contacts to the bound peptide are considered.

Discussion

The HLA-A1:MAGE-A1 (AM) complex, whose structure is described here, has been employed as a prime target for melanoma-specific cytotoxic T cells, using studies *in vitro* as well as *in vivo*.^{17–19} The expression of this antigen is strictly tumor-associated, because the only cells that are able to display HLA class I or II molecules on their surface together with the MAGE-A1 peptide are malignant cells.²⁰ It was therefore of considerable interest to acquire not only cellular reagents, but also soluble molecules directed against this highly specific target. We have previously pointed out²¹ why recombinant, affinity-matured antibodies mimicking TCR specificity such as Hyb3 are preferable to soluble TCR²² or conventional monoclonal antibodies (for example, see Refs. 23–31) that interact in a peptide-dependent manner with a pMHC: although a major obstacle to the application of soluble TCR molecules is to increase their affinity (at least 100-fold), necessitating cumbersome *in vitro*-affinity maturation procedures, the major problem with TCR-like monoclonal antibodies is to obtain them at all. Consequently, very few such reagents are currently available, forcing us to employ recombinant antibodies.^{14,32}

A number of factors that characterize the binding mode of Hyb3 to its target are likely to contribute to the vastly increased affinity (~1000-fold) when compared with a typical interaction between a pMHC and

a natural ligand. This includes strict shape complementarity between the AM complex and Hyb3, associated with burial of large areas of interaction surface together with the exclusion of water molecules that contributes entropically to structural stabilization. Whether the unusual tilt with regard to the main axes of the binding partners (45°) and the diagonal binding mode of Hyb3 (see Fig. 3) play a role as well is unclear, but it is unlikely that a single factor is responsible for the improved affinity of Hyb3 in comparison to that of TCR or KIR.¹⁴ In addition, a Hyb3-induced fit on the structure of HLA-A1 remained to be studied, as the structure of this pMHC in unliganded form was not known before. To shed light on some of these questions, we focus here on conformational changes induced by Hyb3 and natural HLA class I ligands. In comparison to other pMHC structures,¹ the AM complex does not show any unusual features. Possibly, the only conspicuous property is that the structure lacks any particularly remarkable attributes. In the absence of long peptide side chains, the surface which the complex presents to a ligand is rather flat, necessitating strict shape complementarity between the two binding partners and expulsion of water molecules from the interface, as mentioned earlier.

Only four peptide residues are involved in direct interactions with Hyb3, and nearly always peptide main chain atoms are contacted by this ligand.¹⁴ With regard to the HC, five α 1-helix residues (65, 69, 72, 73, 80) as well as three α 2-helix residues (145, 146, 155) engage in H-bonds with Hyb3, whereas three residues (76, 80, 146) are recognized through van der Waals interactions. A comparison of those residues undergoing pronounced conformational changes (>2.0 Å)

Figure 5. Changes in orientation of HLA-A1 residues directly contacting Hyb3 in Hyb3-liganded structure. Panels A and C show the orientation and interactions of residues Arg65, Gln72, Arg145, and Lys146 (shown in gray) in the unbound HLA-A1:MAGE-A1 complex and in the Hyb3-bound complex, where they form direct hydrogen bonds with Hyb3 (panels B and D). The HLA-A1 HC is shown in gray; the Hyb3-H and -L residues in yellow and sky-blue, respectively, and the MAGE-A1 peptide in orange (unbound complex) or green (Hyb3-bound complex). (A) Arg65 forms a hydrogen bond with Gln62 and a water-mediated hydrogen bond with Glu58 in the unliganded structure. Gln72 contacts Arg75 through side chain hydrogen bonds and forms an intrasidic water-mediated hydrogen bond. (B) Arg65 hydrogen bonds with Hyb3 CDR-H1 residues Asp30 and Asp31 in the Hyb3-bound complex. In the unliganded and the liganded structures, the Arg65CZ atoms are separated by 2.73 Å. Gln72 is engaged in contacting Tyr100C and Phe98 of Hyb3-CDR-H1. Its CD atom is separated by 2.32 Å in the two structures. (C) Arg145 forms a network of water-mediated contacts with Gln141 and Met138 in the unbound complex structure, where it also forms a hydrogen bond with a symmetry-related residue (not shown). Lys146 contacts peptide residues pTyr⁹ and pSer⁸. (D) Arg145 contacts CDR-L3 residue Arg94 in the Hyb3-liganded structure. Its side chain apex (CZ atom) is separated by 5.54 Å in the two structures. Lys146 contacts the CDR-L3 residues Ser93 and Asp95A, the MAGE-A1 residue pTyr⁹ and HLA-A1 HC residue Tyr84 through direct hydrogen-bonds. Its NZ apex is separated by 3.60 Å in the two structures.

upon Hyb3 binding (Arg65, Gln72, Arg145, and Lys146; Fig. 5) with comparable reorientations of HC residues in structures of classical pMHC molecules for which information on $\alpha 1/\alpha 2$ -domain-liganded and unliganded forms is available can thus be performed. Apart from the AMF complex, this comparison includes a recently determined second complex between a pMHC and an antibody, nine complexes of pMHC:TCR and one pMHC:KIR structure (Table II, which provides also the respective references). We have refrained from including the HLA-B*3501:EPLP:ELS4 complex in the comparison because of the atypical flattening of the highly bulged 11-mer EPLP peptide which is induced by the binding of the ELS4 TCR.⁴⁹ In 10 of the complexes listed in Table II, pepti-

des in the liganded and unliganded forms overlay with rms deviation values of less than 0.5 Å, indicating that there exist only marginal ligand binding-induced conformational changes for peptide residues. The only elevated rms deviation values (1.01 Å and 0.63 Å) for a bound peptide are noted when the unliganded and the liganded forms of HLA-A2:Tax and H-2K^b:dEV8 are compared (Table II). In the latter case, a reorientation of the side chain of residue pTyr6 appears mainly responsible. A similar overlay of HC residues belonging to the $\alpha 1$ - and $\alpha 2$ -domains of unliganded and liganded pMHC structures gave a much wider spectrum of rms deviation values (Table II). They varied from 0.38 Å (for AMF) to 0.99 Å (for H-2K^b with the pBM1 peptide). Although far-reaching effects of conformational

Table II. Comparison of MHC Class I Structures in Liganded and Unliganded Forms

MHC molecule	HLA-A1	H-2K ^b	HLA-A2	HLA-A2	HLA-A2	HLA-B8	HLA-B*3508	H-2K ^b	H-2K ^b	H-2K ^b	H-2K ^b	H-2K ^b	H-2K ^b	HLA-Cw4
Peptide	MAGE-A1	pOV8	p1049	TCR AHIII2.2	Tax	FLR	LPEP	pKB1	VSV8	pBM1	dEV8	dEV8	dEV8	C4CON1
Ligand	Fab-Hyb3	25-D1.16	TCR AHIII2.2	TCR AHIII2.2	TCR A6	TCR LC13	TCR SB27	TCR KB5C20	TCR BM3.3	TCR BM3.3	TCR 2C	TCR 2C	TCR 2C	KIR2DL1
PDB code, pMHC	3BO8 ^a	1VAC ³³	1BOG ³⁴	1BOG ³⁴	1HHK ³⁵	1MO5 ³⁶	1ZHI ³⁷	1KJ2 ³⁸	2VAA ³⁹	1NAN ⁴⁰	1LEG ⁴¹	1LEG ⁴¹	1LEG ⁴¹	1QOD ⁴²
PDB code, pMHC:ligand	1W72 ¹⁴	3CVH ⁴³	1LP9 ⁴⁴	1LP9 ⁴⁴	1AO7 ¹⁶	1MI5 ⁴⁵	2AK4 ⁴⁶	1KJ2 ³⁸	1NAM ⁴⁰	1FOG ⁴⁷	2CKB ⁴⁸	2CKB ⁴⁸	2CKB ⁴⁸	1IM9 ⁴
pMHC BSASA ^b	990	1134	1193	1193	1021	1135	1609	1482	1058	704	1093	1093	1093	786
Comparisons of pMHC with pMHC:ligand complexes														
HC residues contacting ligand	9	11	9	9	7	15	5	6	7	9	5	5	5	8
HC residues shifting >2.0 Å ^c	4	4	1	1	3	5	4	2	4	1	1	1	2	2
Peptide residues contacting ligand	3	5	6	6	5	2	5	3	2	3	3	3	3	0
Peptide residues shifting >1.0 Å ^d	0	3	0	0	4	1	2	0	1	0	5	5	5	0
Comparisons of rms deviation values (in Å) between pMHC and pMHC:ligand complexes														
Peptide	0.37	0.34	0.24	0.24	1.01	0.28	0.36	0.14	0.14	0.29	0.63	0.63	0.45	0.23
HC ^e	0.38	0.59	0.47	0.47	0.54	0.47	0.73	0.52	0.58	0.99	0.73	0.73	0.66	0.44
β ₂ m	0.32	0.88	0.50	0.50	0.42	0.38	0.37	0.43	1.36	1.66	0.89	0.89	0.75	0.40

^a This study.

^b Solvent accessible surface area of pMHC buried due to binding of its ligand.

^c Ligand-contacting HC residues with side chain apex shift ≥ 2 Å.

^d Peptide residues with side chain apex shift ≥ 1 Å.

^e Only the $\alpha 1$ - and $\alpha 2$ -domains of the HC are compared.

In those cases where more than one molecule within the asymmetric unit is present, only the first molecule has been analyzed.

changes or particular residues cannot be excluded (for e.g., see Ref. 50), changes in the $\alpha 3$ -domain and $\beta 2m$ are not considered further here, because Hyb3, TCR, and KIR do not bind to these parts of a pMHC.

A comparison of the solvent-accessible surface areas that are buried upon binding of a ligand (BSASA values, Table II) demonstrates that these values may reach 1947 Å², but are often only about 1000 Å². Remarkably, both complexes with artificial ligands (AMF and H-2K^b:pOV8:25-D1.16) exhibit typical BSASA values despite their ~ 1000 -fold increased affinity when compared with typical TCR (Table II). Therefore, the BSASA values alone cannot explain the high affinities of antibody molecules that bind pMHC in a TCR-like fashion.

In all nine pMHC:TCR complexes, several HC residues contacting the TCR are affected by changes in side chain orientation. Differences in the orientation of residues 62 (Gln or Arg, 3/9), 65 (Gln or Arg, 5/9), 72 (Gln, 3/9), and 155 (Gln or Arg, 3/9) can most frequently be observed for the TCR-liganded pMHC structures (Table III). For the two published KIR-liganded structures, a comparison is only possible for HLA-Cw4 and KIR2DL1, since a structure of the unliganded HLA-Cw3 is not available. Only two of the HC residues that are contacted by KIR2DL1 undergo conformational changes >2.0 Å: Lys80 and Arg145 (Table III). The degree of reorientation of HC residues that are ligand-contacted is usually ≤ 4.0 Å (27/30 cases), but may reach 6.66 Å (Arg79, in HLA-B8), 5.54 Å (Arg145, in HLA-A1), or 5.30 Å (Arg65, in HLA-A2) (Table III).

With regard to the HC residues undergoing conformational changes, the results presented here demonstrate that Hyb3 recognizes a conformationally active epitope that could be termed “hybrid,” because it is composed of elements which are typically part of footprints on pMHC made by TCR (residues 65 and 72) as well as KIR (residue 145) (Table III).^{2–4} Although it is currently still difficult to derive any generalizations from the few available structures with KIR ligands, residue 145 is clearly undergoing conformational reorientations in complexes of Hyb3 and KIR2DL1, the residues 65 and 72 are part of a triangle-shaped group of amino acids that TCR tend to recognize on the surface of pMHC. In case of class I molecules, it incorporates residues 65, 69, 72 ($\alpha 1$ -helix), and 155 ($\alpha 2$ -helix).² A constellation of residues mimicking 65, 69, and 155 has also been found on MHC class II molecules (residues 57 and 61 of the α -chain and 70 of the β -chain). These residues may be key elements of conserved interactions that TCR engage in when contacting MHC class I or class II antigens during positive and negative selection in the thymus (reviewed recently by Marrack *et al.*⁵¹). Of the conserved HC residues contacted by TCR or Hyb3, one of the KIR molecules contacts the residues 69 and 72.⁴

The results with natural ligands of pMHC lead to the question why a nonnatural ligand, Hyb3, should

Table III. Comparison of Conformational Changes of Selected HC Residues Between Unliganded and Liganded pMHC

Liganded pMHC complex	Ligand-contacting HC residue with apex shifts ≥ 2.0 Å (values in Å)											
	58	62	65	72	79	80	145	146	149	151	154	155
HLA-A1:MAGE-A1:Hyb3			2.7	2.3			5.5	3.6				
H-2K ^b :pOV8:25-D1.16		2.6		2.7	2.4			2.0				
HLA-A2:p1049:AHIII12.2			5.3									
HLA-A2:Tax:A6	2.7		2.2									3.1
HLA-B8:FLR:LC13		3.3	3.4	2.2	6.7							3.8
HLA-B*3508:LPEP:SB27			2.0							3.5	3.1	3.5
H-2K ^b :pKB1:KB5C20		4.0		3.4								
H-2K ^b :VSV8:BM3.3		2.5	3.8					2.3			3.3	
H-2K ^b :pBM1:BM3.3								3.2				
H-2K ^b :dEV8:2C	3.6			2.9								
H-2K ^{bm3} :dEV8:2C			2.4						2.4			
HLA-Cw4:C4CON1:KIR2DL1						3.5	2.7					

rely at least partly also on residues for interaction that are crucial docking points for TCR and KIR. It has been argued that the environments of residues 65, 69, and 155 (class I) as well as those of the corresponding residues of class II antigens permit TCR to interact particularly well with main chain atoms of these amino acids, whereas peptide residues are typically recognized by side chain interactions.^{52,53} Predominantly, Hyb3 employs a different strategy to achieve its specificity: of 13 direct H-bonded contacts ≤ 3.5 Å between HC and Hyb3 residues, only two (with Ala69 and Gln72) are with HC main-chain atoms. Both contacts are very short (2.6 and 2.5 Å, respectively). In contrast, of four such contacts with the MAGE-A1 peptide, only one (with pHis7NE2) is not with a main-chain atom.¹⁴ Very likely, the nonnatural way in which Hyb3 was produced (selection of a low-affinity Fab fragment from a phage display library, phage display-based affinity maturation) releases this reagent from biological constraints that appear to govern the interaction of pMHC with TCR or KIR. For example, it has been proposed that the commonly observed diagonal interaction between pMHC and TCR is influenced by the presence of CD8 molecules within the interaction complex *in vivo*.⁴⁴ It is therefore the more remarkable that Hyb3 shares several residues that are often subject to conformational changes upon binding of natural ligands. It could be that the chemical nature of the side chains of residues 65, 72, and 145 (twice Arg, and Gln in case of the HLA-A1 HC) is particularly well suited for an interaction with ligands, irrespective of whether they are natural or artificial.

Although TCR usually assume a diagonal binding mode, there seems, however, a priori no good reason why Hyb3 should not recognize its target from one of the ends of the peptide-binding groove without loss of specificity and affinity. Even a TCR that interacts via such an unorthodox binding mode has recently been described in case of the nonclassical MHC molecule T22 and the $\gamma\delta$ TCR G8.⁵⁴ In conclusion, the recogni-

tion of HLA-A1 by Hyb3 resembles in many respects those which have been observed for various pMHC and natural ligands, and this is also the case when conformational reorientations of HC residues due to binding of the different ligands are evaluated. A further understanding of the very high affinity between HLA-A1 and Hyb3 may come from structures of unliganded Hyb3 or HLA-A1 complexed with Fab-G8, the low-affinity ancestor of Hyb3.³²

Methods

Protein expression, complexation, and purification

The HLA-A1 HC and β_2m were separately expressed in *Escherichia coli* and purified in the form of inclusion bodies. The MAGE-A1 peptide (EADPTGHSY) was purchased from Alta Biosciences, UK. Inclusion bodies of HLA-A1 HC and β_2m were unfolded, mixed with the MAGE-A1 peptide in a refolding buffer (1:2:10 molar ratio), and reconstituted for 7–10 days at 4°C to form the HLA-A1: β_2m :MAGE-A1 complex (AM) as detailed previously.^{32,55} The complex was purified by size exclusion chromatography on a Superdex 75HR gel filtration column (Amersham Biosciences) using a pH 7.5 buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 0.1% NaN₃, concentrated to 15–17 mg/mL and used for crystallization screens.

Crystallization and data collection

The AM complex was crystallized using the PEG-ion screen of Hampton Research, USA in a sitting drop vapor diffusion setup at 18°C, with a reservoir volume of 100 μ L and drops made up of 1.1 μ L protein and 1.1 μ L reservoir solution. A single AM crystal was obtained in a well containing 20% PEG3350 and 0.2 M NaF as reservoir after 18 days. Visible satellite crystals were removed by cutting the crystals into smaller pieces. Following flash-cooling in liquid nitrogen after brief soaking in a cryo-buffer composed of

reservoir and 15% glycerol, X-ray diffraction data was collected at Protein Structure Factory beamline BL-1 of Freie Universität Berlin installed at the BESSY II synchrotron in Berlin. The crystal diffracted to a resolution limit of 1.8 Å, and the collected X-ray data was indexed and integrated using MOSFLM,⁵⁶ and then scaled and merged using program SCALA.⁵⁷

Structure determination and analysis

The HLA-A1 molecule was localized in the crystal unit cell by molecular replacement using programs MOL-REP⁵⁸ and PHASER,⁵⁹ with HLA-A1:MAGE-A1:Hyb3 (PDB code 1W72) as search model from which Hyb3, water molecules, and peptide were stripped off. The obtained model was subjected to iterative cycles of restrained-maximum likelihood refinement including isotropic temperature factor adjustment using REFMAC,⁶⁰ followed by manual rebuilding using COOT.⁶¹ Water molecules were positioned using CNS.⁶² The SASA and BSASA of residues of the structures were calculated with AREAIMOL⁶³ using a probe radius of 1.4 Å. All superpositions and root mean square (rms) deviation calculations were performed using LSQKAB.^{64,67}

The atomic coordinates and structure factors (code 3BO8) have been deposited in the Protein Data Bank (www.pdb.org).

Preparation of 2D and 3D figures

The 2D figures showing structural details were prepared using PyMOL.⁶⁵ To view conformational changes and further differences between the two structures (see 3D comparison of the AM and the AMF structures in the results section), we employ here, for the first time in a novel description of the structure of a protein, fully interactive three-dimensional (3D) representations in the electronic version of this article that were created using PyMOL and Adobe Systems' "Acrobat 9 Pro Extended" software. The procedure to create 3D models of a molecular complex has recently been described.⁶⁶ Initially, the raw PDB files (3BO8 and 1W72) were opened using PyMOL version 1.0r1. The components to be shown in the final 3D model were selected and each exported separately as VRML2 worlds with appropriate designations (e.g., beta2m.wrl) followed by import into Adobe 3D Reviewer (Adobe Systems, San Jose, CA). The model, complete with all components, was exported as a PDF model with tessellation compression export format. The PDF model was then opened in Adobe Acrobat 9 Pro Extended and an object hierarchy (i.e., model tree) created. Different colors were then assigned to each component followed by saving the complete model as a PDF file.

The three images shown in Figure 3(A–C) as 2D images ("posters") were created from TIFF format desktop screenshots of the desired model views and modified in Corel Draw 11 to show the desired information (i.e., cropped to size, etc) and exported to PDF format as a single file. The previously saved PDF

model was opened in Adobe Acrobat 9 Pro Extended and the 2D PDF poster included in the model. After combining the 2D and 3D files, all final viewing settings were fixed, and preset views were selected using the camera tool followed by defining a standard view that would be shown after activation of the 3D mode. Finally, the model was saved as a PDF file. A step-by-step guide to creating PDF-integrated 3D models is available at www.charite.de/immunogenetik/model3d.

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