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Supine Orientation of a Murine MHC Class I Molecule on the Membrane Bilayer

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Summary

Structural studies of cellular immune receptors such as MHC molecules, T cell receptors (TCR), and TCR/MHC complexes have been carried out with recombinant, soluble forms of the extracytoplasmic domain of these glycoproteins [1]. The important role of the membrane bilayer in T cell recognition and antigen presentation has become increasingly obvious with the description of lipid microdomains [2, 3]. These rafts appear to regulate recognition and signaling by clustering receptors and facilitating the formation of the immune synapse [4]. However, the interactions and orientation of these receptors at the lipid bilayer are unknown. We have used H-2K^b, a major-histocompatibility (MHC) class I molecule, and tethered its soluble domain to a lipid bilayer via a surrogate connecting peptide to reveal the disposition of MHC molecule on the membrane surface. We demonstrate that the long axis of the MHC molecule is approximately parallel to the plane of the membrane with the peptide binding pocket close to the membrane surface. This result was determined by analyzing 4.5Å resolution electron crystallographic projection data from frozen-hydrated 2-dimensional crystals. Ionic interactions between the lipid headgroup and the protein appear to be responsible for this orientation, which could establish a "fourth dimension" during MHC/T cell receptor interactions critical for activation.

Results and Discussion

To date, protein/lipid interactions have been studied mostly in the context of molecules that are involved in lipid metabolism, such as phopspholipase A2 [5, 6]. In order to determine the spatial dispositions of MHC and TCR molecules with respect to the lipid leaflets, we have developed a strategy in which recombinant, soluble forms of these molecules were anchored to synthetic lipids [7]. Briefly, the extracellular domains, extending up to the putative transmembrane boundary and retaining a C-terminal histidine tag, were expressed and purified. A nickel-chelating lipid then captured the histidine tag to reattach the molecule to a lipid bilayer [7] and mimic the normal in vivo display. The membrane bound molecules were fully functional and accessible to their natural ligand and to antibody binding [7]. Two-dimensional crystals of lipid-tethered H-2K^b were previously generated by using this technique and preserved in uranyl acetate to produce a 3-dimensional structure of the membrane-anchored molecule by electron microscopy. The resolution of this map, as deduced by us now, using the point-spread-function criterion [8] is nominally $\sim$14Å in plane and $\sim$24Å normal to the membrane plane. Notwithstanding the low resolution of this map, it was shown by Celia et al. [7] to agree qualitatively with the commonly believed orientation of the MHC molecule with respect to the lipid bilayer.

To reveal unambiguously the orientation of the H-2K^b molecule with respect to the membrane surface, the crystals were preserved unstained in vitrified buffer [9] and used to record and analyze high-resolution electron crystallographic data in projection. The experimentally determined density map (Figure 1A) revealed a view of the membrane-anchored H-2K^b molecule projected onto the membrane plane; however, the assignment of density to α1, α1 and α3, and β2 microglobulin domains and, hence, the orientation in three dimensions was not immediately obvious. This correspondence was elucidated by molecular replacement calculations on the electron crystallographic data by using the atomic coordinates derived from the X-ray structure [10]. For this calculated orientation, a "model" projection density map (Figure 1B) was generated, which corresponded well with the experimental map (Figure 1A), confirming the validity of the approach. This analysis demonstrates that 3D orientation information can be successfully determined even from 2D projection data by molecular replacement technique.

The deduced orientation (Figure 1E) and the model map eliminated the ambiguity in identifying the various domains from the experimental map alone because of the relative lack of projected density in some of the linker regions, which, in part, made it difficult to correctly assign the orientation in our earlier [7] low-resolution analysis. The incorrect orientation was approximately orthogonal to that presented here; however, we noted that, the low-resolution three-dimensional volume map that we calculated previously [7] agreed equally well with the current orientation (Supplemental Data). This deduced orientation with respect to the membrane bilayer positioned the MHC molecule on its "side" rather than "standing up," as usually represented, while placing the peptide binding groove approximately perpen-

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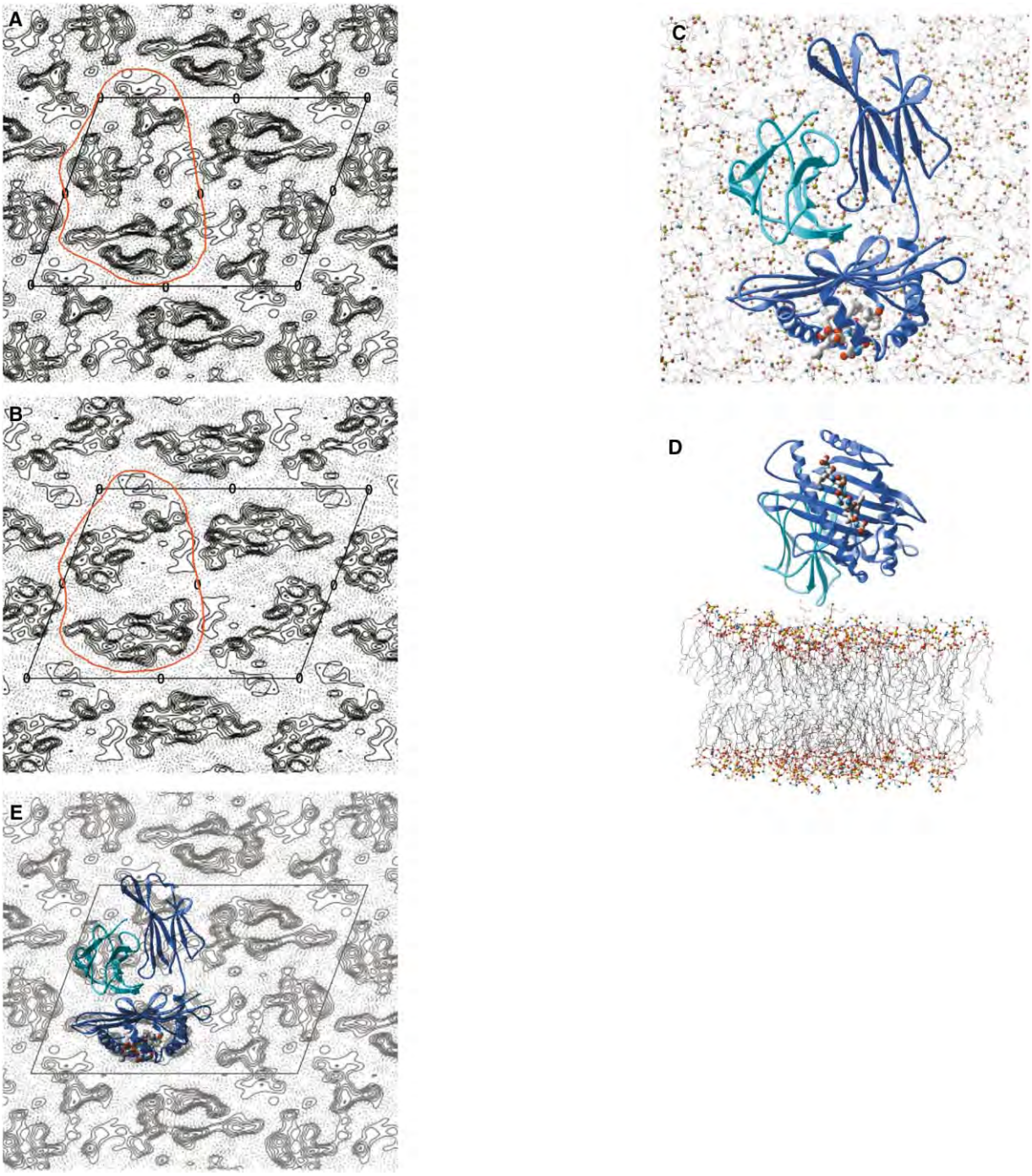

Figure 1. Analysis of the Structure of H-2K^b Molecules Bound to a Lipid Bilayer

(A) The projection-density map of lipid-anchored H-2K^b at 4.5Å resolution determined from electron-crystallographic analysis of frozen-hydrated 2D crystals (plane group symmetry p2) preserved in vitrified buffer.

(B) Model projection-density map calculated at 4.5Å resolution based on the deduced orientation of the H-2K^b molecule in the 2D crystal.

(C) A view of the observed orientation of the H-2K^b molecule projected on to the membrane bilayer.

(D) A view orthogonal to that shown in (C).

(E) Experimental projection map overlaid with the X-ray model in the deduced orientation.

In (A) and (B), one unit cell of the p2 lattice is shown, and the continuous and dashed contours, respectively, represent regions of high (positive) and low (negative) density; the red line approximates the boundary of H-2K^b molecule seen in projection. (C) and (D) were generated by using the AVS software [22] and also by using a model of POPC lipid bilayer [23]. The lipid bilayer is positioned approximately to accommodate the modeled orientation of the sugar moiety attached to Asn176 and the transmembrane C-terminal α-helix (see Figures 2D and 4).

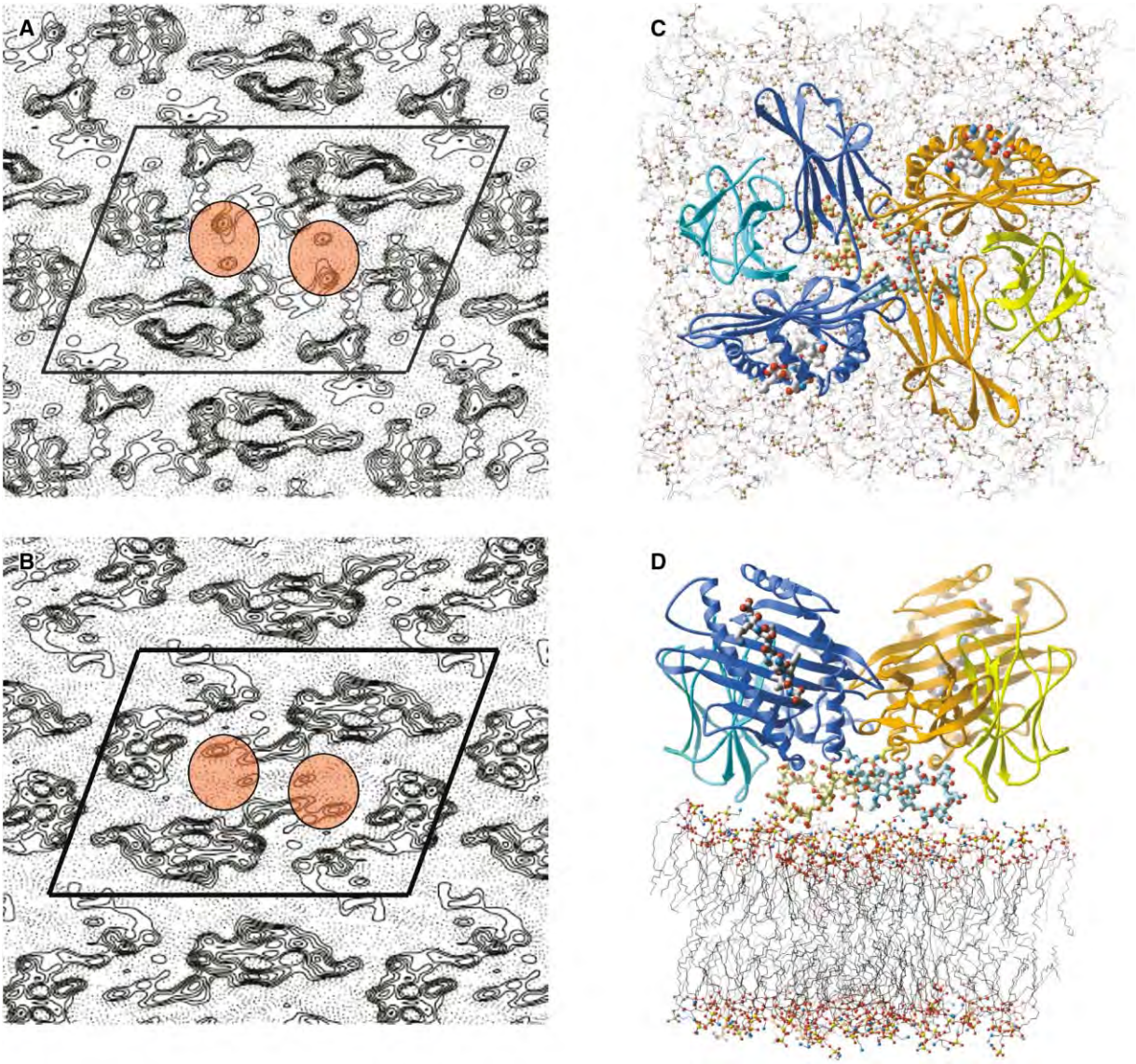

Figure 2. Determination of the Sugar Orientation in the Membrane Bound H-2K^b Structure

(A) The stretch of density (circumscribed by red shadow) attributed to the sugar moiety attached to Asn176, as revealed in the projection density map of lipid-anchored H-2K^b.

(B) Model projection-density map calculated by using the deduced orientation of H-2K^b with an N linked sugar moiety ([α-D-Man]$_9$-[β-GlcNAc]$_2$) attached to Asn176. A qualitative agreement between the shaded additional density seen in this map and that in experimental projection map (A) can be seen.

(C) Projected view of the H-2K^b dimer with the modeled sugar moiety.

(D) The view orthogonal to that shown in (C).

dicular to the membrane surface. A similar orientation for surface MHC-like molecules was suggested earlier for the FcRn receptor, which binds dimers of the Fc portion of immunoglobulin [11]. The 2D crystal lattice, characterized by p2 plane-group symmetry, contains two membrane-anchored H-2K^b molecules, called hereafter an H-2K^b dimer. The stabilization of these dimers is brought about by intermolecular contacts across the 2-fold axis perpendicular to the lipid plane. There could also be a potential role of glycosylation at Asn176 and Asn86 in the observed packing of the MHC molecules. We found, for instance, that the strong density peaks noted near the central 2-fold axis (Figure 2A), which were proximal to symmetry-related Asn176 in the α2 domain, were not present in the model map (Figure 1B) derived from the X-ray structure [10] where the sugar groups were not visualized. Assuming no substantial conformational changes in H-2K^b molecule upon tethering to the membrane surface, these peaks then suggest the location of the sugar moiety, which could participate in additional packing interactions stabilizing the MHC dimers in the deduced orientation. In fact, using a model for the N linked sugar structure [12] and noting that due to close packing near the 2-fold axis the sugar moiety attached to Asn176 could only be oriented approximately orthogonal to the membrane surface, we

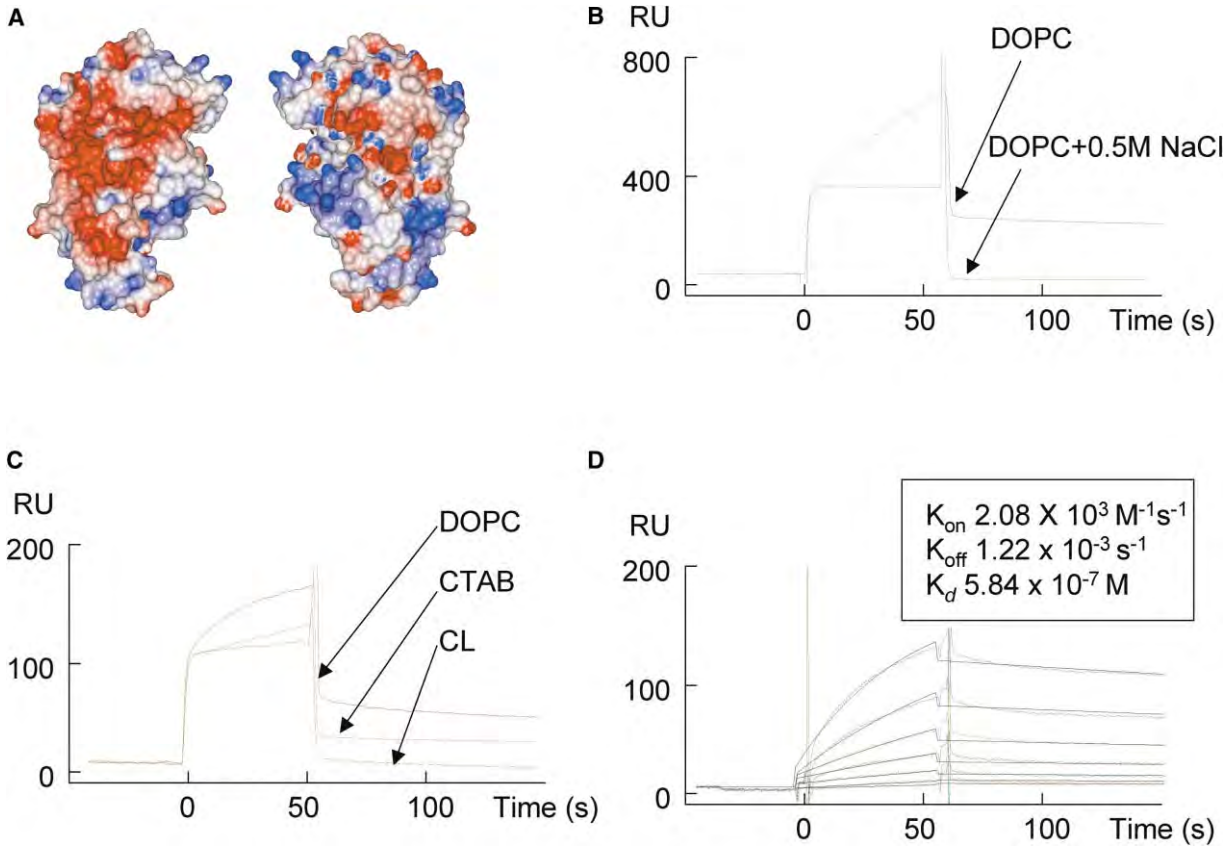

Figure 3. Ionic Interactions Drive the Binding of H-2K^b to Phospholipids

(A) Surface electrostatics of H-2K^b. Electrostatics was calculated with the Delphi module within Insight II (Biosym Technologies, San Diego, CA). Formal charges were assigned to the protein coordinates. Positive charges are contoured blue, while negative charges are contoured red (-5 to $+5$ kT/e). β2 microglobulin is on the inside of the two objects, which are rotated 180° respective to each other along the vertical axis.

(B) The binding of H-2K^b molecules to DOPC surface is abrogated in the presence of high salt concentrations. H-2K^b molecules were injected over a DOPC surface in either PBS- or 0.5 M NaCl-containing buffer. Sensorgrams for buffer alone were subtracted from the respective H-2K^b traces.

(C) Relative binding of H-2K^b molecules to DOPC, CTAB, and CL surfaces. H-2K^b at a single 10 μM concentration was injected over three successive flow cells coated with DOPC, CTAB, and CL, respectively. The traces for buffer alone were subtracted from each sensorgram.

(D) The affinity of H-2K^b to DOPC was measured to be in the low micromolar range. H-2K^b molecules were injected over a DOPC surface in PBS at 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0.15625 μM. Traces for buffer alone were subtracted from each sensorgram. A second subtraction from the sensorgram at each concentration was carried out to remove background refractive index (sensorgrams in buffer containing 0.5M NaCl) in order to measure actual binding. The curves were analyzed by global fitting by using the BIAevaluation 3.01 software.

were able to explain the aforementioned density (Figure 2B). In this orientation the sugar moiety has a large, interacting surface with the membrane, which may be relevant in vivo because measurement of the diffusion coefficient for MHC molecules with and without sugars showed that glycosylation limited the translational D coefficient [13]. This observation suggested that sugars were interacting with membrane and/or neighboring molecules.

As noted above, the deduced orientation of the H-2K^b molecule on the surface of the lipid layer implies that a large, interacting surface is elaborated between the protein and the lipid head groups. Most outer-cell surface lipids are phospholipids that display a zwitterionic head group with a proximal negatively charged phosphate group and a distal positively charged nitrogen. Charge and accessibility of the different parts of the head groups depend on the lipid composition and on the pH of the extracellular medium. Ionic interactions between membranes and proteins have been shown in a number of systems, such as AFABP [14], cytochrome C [15], and protein kinase C [16]. Evaluation of the electrostatic charge distribution on the surface of the H-2K^b revealed that the major faces have uneven charge distribution with large, negatively-charged central patches, while scattered positively-charged patches characterize the periphery (Figure 3A). A series of surface plasmon resonance (SPR) experiments were carried out to probe possible interaction of H-2K^b with lipid membranes under physiological conditions. The H-2K^b molecules interacted strongly with di-oleoyl phosphatidylcholine (DOPC) (Figure 3B), weakly with the neutral quaternary ammonium cetyltrimethylammonium bromide (CTAB), and imperceptibly with negatively charged cardiolipin (CL) (Figure 3C). H-2K^b/DOPC interaction was measured in the low micromolar range by injecting successive dilutions

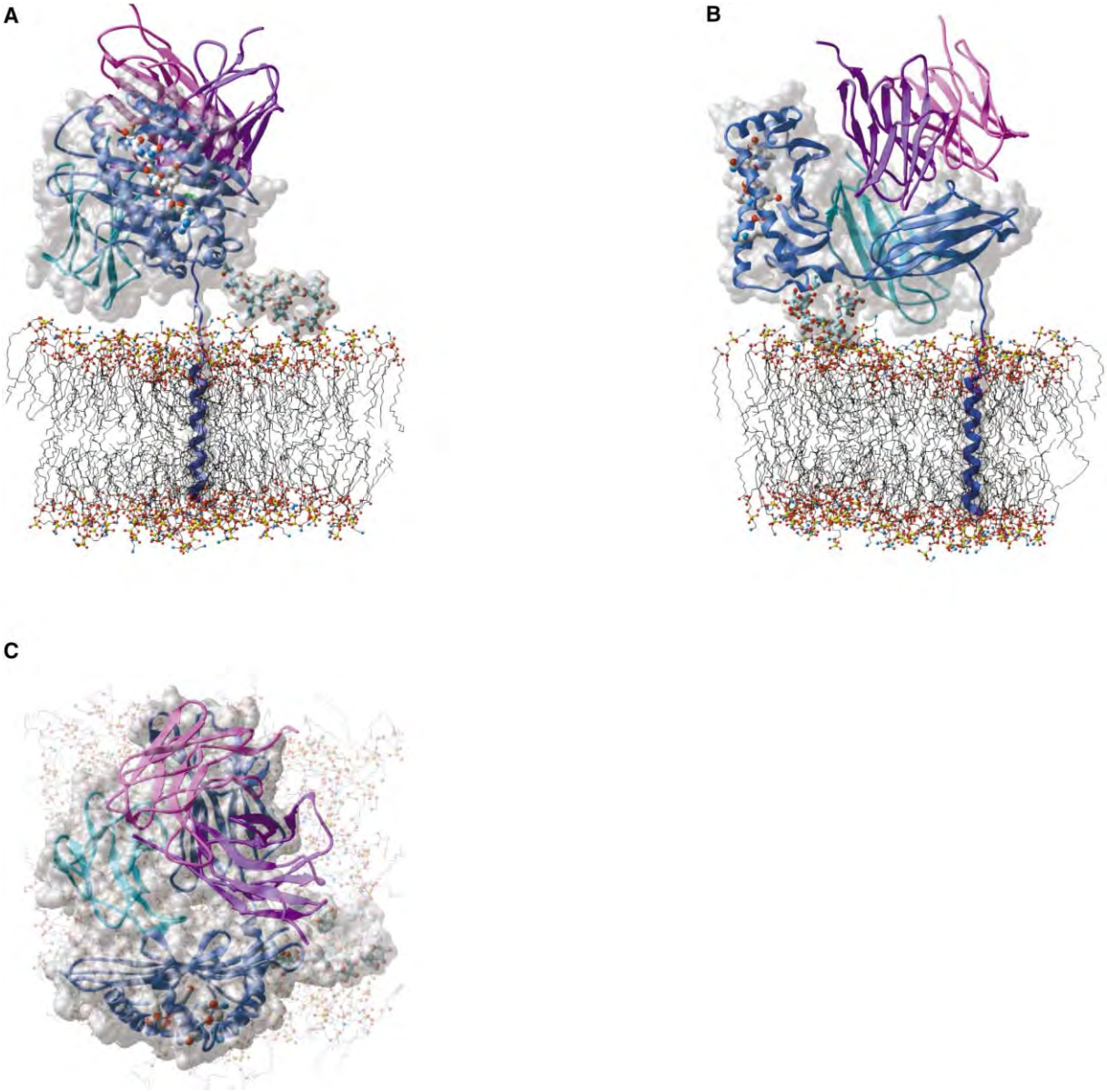

Figure 4. The Membrane-Anchored H-2K^b Molecule Is Fully Accessible to CD8 Binding

(A) Front view, (B) side view, and (C) top view. CD8 is represented in ribbon diagram (purple), whereas H-2K^b is represented with surfaces. The connecting peptide and the transmembrane segment have been added schematically to the X-ray structure of H-2K^b as a β strand and a α helix, respectively, without any structural information except their known lengths. The lipid bilayer is a POPC membrane, as in Figures 1 and 2. Images were generated by using AVS software [22].

of the MHC, and the observed interaction was abrogated when the salt concentration was raised to 0.5 M (Figure 3B), establishing that the interaction was ionic in nature. We concluded that anionic interactions between the negatively charged surface of the molecule and the nitrogen of the lipid head group were primarily responsible for the observed preferred orientation of H-2K^b. Similar results were obtained with H-2K^b molecules from which sugars had been partially removed by mannosidase treatment or totally removed by being produced in the presence of tunicamycin (data not shown). This observation suggests that if sugars were important for the packing of the crystals, they do not appear to impact the ionic interaction of the MHC with the lipid headgroups. The nature and the segregation of lipids in lipid rafts and pH can influence such interaction under physiological conditions. For instance, T cell activation is followed by acidification of the extracellular medium [17] and by the partitioning of surface receptor into rafts at the surface of both T cells and antigen-presenting cells. In the orientation that is presented here, accessibility of binding sites on MHC for both TCR and accessory molecules needed to be examined in order to evaluate functional consequences. We had shown previously that soluble TCR binds lipid-anchored MHC with normal kinetics [7]. Similarly, Fab fragments of an anti-α1α2 domain anti-

body [18] bound lipid-anchored H-2K^b with similar kinetic parameters when compared to their binding to randomly immobilized MHC (Table S2). However, intact immunoglobulin of the same antibody exhibited a slower on rate but an identical off rate on lipid-bound MHC as compared to MHC-coupled to the chip in random orientation (Table S2). This difficulty for the whole Ig to engage its two sites to produce cooperative binding is consistent with the orientation of the MHC molecule described here. We also assessed the accessibility of the lipid-bound H-2K^b to the CD8 molecule by substituting the H-2K^b molecule for the H-2K^b $-$CD8$\alpha\alpha$ complex [19] by using the deduced orientation. As can be seen from Figure 4, CD8 has full access to its binding site at the α2-α3 boundary of H-2K^b and could engage in its accessory function. In this orientation the stalk region of CD8 does not have to be bent to engage the immunoglobulin-like domains of CD8 to MHC binding, as often represented in schematic diagram of T cell activation. Similarly, this orientation also gives full access for NK receptors such as Ly49A on H-2D^d [20] and Ly49C on H-2K^b [21] to bind to the MHC molecule.

What exactly are the molecular switches that trigger the T cell activation process? The observed orientation of H-2K^b on the lipid bilayer in combination with the optimal accessibility to CD8 binding raises the exciting possibility that the interaction of MHC with the membrane plays a critical role in TCR interaction. Does the interaction with the TCR trigger reorientation of the MHC on the lipid surface, or conversely, does the MHC impose a reorientation of the TCR within its own multimolecular complex after binding and, hence, trigger activation? It can also be argued that accessory molecules would also play a role in lifting MHC molecules from the membrane, allowing simultaneous interaction of TCR with MHC and CD4/CD8. Our findings of MHC interactions with the membrane set the stage for further investigation of the dynamics of T cell activation on the surface of living cells.

Experimental Procedures

Crystallization, Electron Microscopy, and Data Processing

Two-dimensional crystals of 6-His-tagged H-2K^b generated on lipid monolayer containing DOGS-NTA-Ni lipid as described [7] were transferred onto hydrophobic holey carbon films (Quantifoil, P1 Supplies, PA) and then flash frozen. Minimal dose images of vitrified crystals were recorded by using a Philips CM200FEG microscope operated at 120KV, and electron diffraction patterns were collected on a Philips CM120 microscope operated at 100KV. The crystal lattice conformed to the plane group of symmetry p2 ($a = 92.0$ Å, $b = 68.0$ Å, $\gamma = 113^\circ$). Reflections up to 4.5Å resolution with CTF-corrected phases (Table S1) and derived from merging of five images and amplitudes derived from merging of two electron diffraction patterns comprised the projection data set. The MRC software package [24] was used for data processing and analysis.

Molecular replacement calculations (results shown in Table S1) were carried out by using the MOLREP (CCP4 package [25]) program. For this purpose, the projection amplitudes and the experimentally determined phases (centro-symmetric) were used, and the X-ray structure of soluble H-2K^b-ovalbumin peptide complex [10] (Protein Data Bank coordinate 1VAC) served as the search model.

Model projection density maps from the atomic coordinate files were computed by using the program SFALL in CCP4 package and used only those reflections that comprised the experimental projection data set. A rigid model for the N linked sugar molecule [12] was used for deriving the location and orientation of the N-glycosylated sugar moiety attached to Asn176.

Surface Plasmon Resonance

Surface plasmon resonance measurements were carried out on a BIAcore 2000 (BIAcore, Uppsala). Planar-supported membranes were made by successive 100 μl injections of liposomes (0.25mg/ml) onto HPA sensor chips at a 5 μl/min flow rate. Potential multilamellar structures were rinsed off overnight by a continuous 20 μl/ml flow of PBS. Interaction of purified MHC molecules was measured after removal of the histidine tag by carboxypeptidase A digestion (1:100 w/w ratio) and Ni-NTA chromatography. MHC molecules were injected in PBS or in 0.5M NaCl. Buffer alone sensorgrams were subtracted from MHC-buffer sensorgrams. To evaluate MHC interactions with lipid head groups a secondary set of subtractions was done to remove the MHC refractive index (calculated from the subtractions done with the 0.5M NaCl sensorgrams).

For antibody binding, MHC molecules were captured on lipid surfaces retaining 10% DOGS-NTA lipids, and antibody (whole Ig or Fab) was injected onto that surface. Two-fold dilutions from 1000 nM to 30 nM of the antibody were injected (20 μl/min) to calculate affinity constants. Injections on a noncoated channel were used for subtraction of the sensorgrams. For affinity binding studies on random-coupled MHC, H-2K^b molecules were immobilized by amine coupling on CM5 chip using classical chemistry. Similar injections of the antibody were carried out.

For kinetics studies, all subtracted sensorgrams were analyzed by global fitting by using the BIAevaluation 3.1 software program.

Supplemental Data

Supplemental Data including two tables and four figures are available at http://www.current-biology.com/cgi/content/full/14/8/718/DC1/.

Acknowledgments

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References

1. Garcia, K.C., Teyton, L., and Wilson, I.A. (1999). Structural basis of T cell recognition. Annu. Rev. Immunol. *17*, 369–397.
2. Viola, A. (2001). The amplification of TCR signaling by dynamic membrane microdomains. Trends Immunol. *22*, 322–327.
3. Vogt, A.B., Spindeldreher, S., and Kropshofer, H. (2002). Clustering of MHC-peptide complexes prior to their engagement in the immunological synapse: lipid raft and tetraspan microdomains. Immunol. Rev. *189*, 136–151.
4. Dustin, M.L., Bromley, S.K., Davis, M.M., and Zhu, C. (2001). Identification of self through two-dimensional chemistry and synapses. Annu. Rev. Cell Dev. Biol. *17*, 133–157.
5. Lin, Y., Nielsen, R., Murray, D., Hubbell, W.L., Mailer, C., Robinson, B.H., and Gelb, M.H. (1998). Docking phospholipase A2 on membranes using electrostatic potential-modulated spin relaxation magnetic resonance. Science *279*, 1925–1929.
6. Pan, Y.H., Yu, B.Z., Singer, A.G., Ghomashchi, F., Lambeau, G., Gelb, M.H., Jain, M.K., and Bahnson, B.J. (2002). Crystal structure of human group X secreted phospholipase A2. Electrostatically neutral interfacial surface targets zwitterionic membranes. J. Biol. Chem. *277*, 29086–29093.
7. Celia, H., Wilson-Kubalek, E., Milligan, R.A., and Teyton, L. (1999). Structure and function of a membrane-bound murine MHC class I molecule. Proc. Natl. Acad. Sci. USA *96*, 5634–5639.
8. Unger, V.M., and Schertler, G.F. (1995). Low resolution structure

of bovine rhodopsin determined by electron cryo-microscopy. Biophys. J. *68*, 1776–1786.

9. Ren, G., Cheng, A., Reddy, V., Melnyk, P., and Mitra, A.K. (2000). Three-dimensional fold of the human AQP1 water channel determined at 4 A resolution by electron crystallography of two-dimensional crystals embedded in ice. J. Mol. Biol. *301*, 369–387.
10. Fremont, D.H., Stura, E.A., Matsumura, M., Peterson, P.A., and Wilson, I.A. (1995). Crystal structure of an H-2K^b-ovalbumin peptide complex reveals the interplay of primary and secondary anchor positions in the major histocompatibility complex binding groove. Proc. Natl. Acad. Sci. USA *92*, 2479–2483.
11. Burmeister, W.P., Huber, A.H., and Bjorkman, P.J. (1994). Crystal structure of the complex of rat neonatal Fc receptor with Fc. Nature *372*, 379–383.
12. http://www.dkfz-heidelberg.de/spec/sweet2/doc/index.html/.
13. Wier, M., and Edidin, M. (1988). Constraint of the translational diffusion of a membrane glycoprotein by its external domains. Science *242*, 412–414.
14. Smith, E.R., and Storch, J. (1999). The adipocyte fatty acid-binding protein binds to membranes by electrostatic interactions. J. Biol. Chem. *274*, 35325–35330.
15. Salamon, Z., and Tollin, G. (1996). Surface plasmon resonance studies of complex formation between cytochrome c and bovine cytochrome c oxidase incorporated into a supported planar lipid bilayer. II. Binding of cytochrome c to oxidase-containing cardiolipin/phosphatidylcholine membranes. Biophys. J. *71*, 858–867.
16. Bittova, L., Stahelin, R.V., and Cho, W. (2001). Roles of ionic residues of the C1 domain in protein kinase C-alpha activation and the origin of phosphatidylserine specificity. J. Biol. Chem. *276*, 4218–4226.
17. Beeson, C., Rabinowitz, J., Tate, K., Gutgemann, I., Chien, Y.H., Jones, P.P., Davis, M.M., and McConnell, H.M. (1996). Early biochemical signals arise from low affinity TCR-ligand reactions at the cell-cell interface. J. Exp. Med. *184*, 777–782.
18. Ajitkumar, P., Geier, S.S., Kesari, K.V., Borriello, F., Nakagawa, M., Bluestone, J.A., Saper, M.A., Wiley, D.C., and Nathenson, S.G. (1988). Evidence that multiple residues on both the alpha-helices of the class I MHC molecule are simultaneously recognized by the T cell receptor. Cell *54*, 47–56.
19. Kern, P.S., Teng, M.K., Smolyar, A., Liu, J.H., Liu, J., Hussey, R.E., Spoerl, R., Chang, H.C., Reinherz, E.L., and Wang, J.H. (1998). Structural basis of CD8 coreceptor function revealed by crystallographic analysis of a murine CD8alphaalpha ectodomain fragment in complex with H-2K^b. Immunity *9*, 519–530.
20. Tormo, J., Natarajan, K., Margulies, D.H., and Mariuzza, R.A. (1999). Crystal structure of a lectin-like natural killer cell receptor bound to its MHC class I ligand. Nature *402*, 623–631.
21. Dam, J., Guan, R., Natarajan, K., Dimasi, N., Chlewicki, L.K., Kranz, D.M., Schuck, P., Margulies, D.H., and Mariuzza, R.A. (2003). Variable MHC class I engagement by Ly49 natural killer cell receptors demonstrated by the crystal structure of Ly49C bound to H-2K^b. Nat. Immunol. *4*, 1213–1222.
22. Upson, C., Faulhaber, T., Jr., Kamins, D., Laidlaw, D., Schlegel, D., Vroom, J., Gurwitz, R., and Van Dam, A. (1989). The application visualization system: a computational environment for scientific visualization. Comput. Graph. Appl. *9*, 30–42.
23. Tieleman, D.P., Berendsen, H.J., and Sansom, M.S. (1999). An alamethicin channel in a lipid bilayer: molecular dynamics simulations. Biophys. J. *76*, 1757–1769.
24. Crowther, R.A., Henderson, R., and Smith, J.M. (1996). MRC image processing programs. J. Struct. Biol. *116*, 9–16.
25. CCP4 (Collaborative Computational Project, Number 4) (1994). The CCP4 suite: programs for protein crystallography. Acta Crystallogr. D Biol. Crystallogr. *50*, 760–763.