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Crystal structure of the *Mycoplasma arthritidis*-derived mitogen in apo form reveals a 3D domain-swapped dimer

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SUMMARY

Mycoplasma arthritidis -derived mitogen (MAM) is a superantigen (SAG) that can activate large fractions of T cells bearing particular V β elements of T-cell receptor (TCR). Here we report the crystal structure of a MAM mutant K201A in apo form (unliganded) at 2.8Å resolutions. We also partially refined the crystal structures of the MAM wild-type (WT) and another MAM mutant L50A in apo forms at low resolutions. Unexpectedly, the structures of these apo-MAM molecules display a 3D domain-swapped dimer. The entire C-terminal domains of these MAM molecules are involved in the domain-swapping. Functional analyses demonstrated that the K201A and L50A mutants do not show altered ability to bind to their host receptors, and that they stimulate the activation of T-cells as efficiently as does the wild-type. Structural comparisons indicated that the “reconstituted” MAM monomer from the domain-swapped dimer displays large differences at the hinge regions from the MAM_{wt} molecule in the receptor-bound form. Further comparison indicated that MAM has a flexible N-terminal loop, implying that conformational changes could occur upon receptor binding.

Keywords

Superantigen; *Mycoplasma arthritidis* mitogen; 3D domain-swap; crystal structure

Superantigens (SAGs) are immunoregulatory proteins produced generally by bacteria and viruses.^{1; 2} Upon their binding to the host major histocompatibility complex (MHC) class II molecules, SAGs are recognized by T-cell receptor (TCR) in a V β - restricted fashion, resulting in polyclonal activation of large pools of T lymphocytes (up to 20%). *Mycoplasma arthritidis*, a natural pathogen of rodents, produces a soluble T cell mitogen (MAM), which is also a potent SAG.³ MAM can induce spontaneous chronic arthritis that resembles human rheumatoid arthritis, in genetically susceptible strains of rodents. Although MAM functions as a conventional SAG, it does not show significant global sequence homology with other SAGs.⁴

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In order to elucidate the structural basis of T cell activation by this unique SAg MAM, we previously determined the three-dimensional (3D) structure of MAM complexed with one of its host receptors, a human class II MHC molecule, HLA-DR1, loaded with a haemagglutinin peptide 306–318 (HA).⁵ The crystal structure showed that the receptor-bound MAM adopted a fold composed of two completely α -helical domains.⁵ This fold is entirely different from those of other SAGs with known structures.^{1; 6}

More recently, we also determined the first crystal structure of a complete ternary complex of the superantigen MAM complexed with its two receptors, class II MHC HLA-DR1 and TCR.⁷ In that ternary complex structure, receptor-bound MAM retained the conformation seen in the MAM-pMHC binary complex structure.⁵ In addition, the structure of the TCR-MAM-pMHC ternary complex provided the first structural evidence that SAg can directly contact the TCR α chain. Such a direct interaction is striking, since it contradicts an earlier assumption that a hallmark of SAGs is their specificity for the TCR V β domain.^{1; 2}

Three-dimensional domain swapping, defined as the noncovalent exchange of an identical structural element between protein molecules, has been proposed as one of the mechanisms for evolution of protein oligomers.^{8; 9} It has also been hypothesized to play roles in disease-related protein aggregation.^{9; 10; 11}

In the present study, we report the crystal structure of an MAM mutant (K201A) in apo form at 2.8 Å resolution. We also partially refined the crystal structures of the MAM wild-type (MAM_{wt}) and a MAM_{L50A} mutant at low resolution. Our results show that, although these apo MAM molecules were crystallized in different crystal forms, they all display 3D domain-swapped dimers when not bound to their receptors, pMHC and TCR. Structure comparisons showed that the N-terminal four-helical bundles are in similar conformations between receptor-bound and unbound (apo) forms of MAM. However, unlike the receptor-bound MAM monomer of which the C-terminal domain (CTD) folded towards its own N-terminal domain (NTD) forming a “stable” monomer structure,^{5; 7} two apo MAM_{wt} (or mutant) molecules exchanged their CTDs with each other to form a 3D domain-swapped dimer. We further show that the K201A and L50A mutants not only retain their ability to bind to their host receptors, but they also activate T-cells as efficiently as does the wild type. Structural comparisons indicated that the “reconstituted” MAM monomer displays large conformational differences at the hinge regions from the MAM molecule in the receptor-bound form. Further comparison indicated that MAM has a flexible N-terminal loop, implying that conformational changes could occur upon receptor binding.

Structure Solution

Crystals of the wild-type MAM molecule (MAM_{wt}) were obtained first. However, these crystals diffracted poorly and anisotropically.¹² Therefore, several MAM mutants, L50A, R154A, R192A, E195A, and K201A, were produced and subjected for crystallization trials.¹² These residues are located at the interface of a MAM dimer observed in the co-crystal structure of the MAM-pMHC/HA binary complex;⁵ and mutants were originally prepared to study the dimerization of MAM.^{12; 13} Under very similar conditions, two mutants, namely MAM_{K201A} and MAM_{L50A}, gave usable crystals.¹² However, it is noted that MAM_{K201A} was crystallized in a form different from those of MAM_{wt} and MAM_{L50A} (Table 1).¹² Due to these differences in crystal forms, cell content analyses indicated that there are a much smaller number of molecules to occupy an asymmetric unit cell in the MAM_{K201A} crystal than in the MAM_{wt} and MAM_{L50A} crystals (Table 1).¹² As a consequence, the diffraction of the new MAM_{K201A} crystal was greatly improved. Diffraction data up to 2.8 Å resolution was collected for the MAM_{K201A} crystal, whereas the MAM_{wt} and MAM_{L50A} crystals could only diffract to 4.0 Å, and 3.6 Å resolutions, respectively (Table 1).

We solved the structures of these MAM molecules by molecular replacement (MR) with the program PHASER¹⁴ (Fig. 1 and Table 1). With the crystal structure of MAM in the MAM/HLA-DR1/HA complex (PDB; 1R5I)⁵ used as the search model, MR calculations for the MAM_{K201A} data failed to generate a clear solution. The MAM model was therefore divided into two independent domains, with various truncations at the N- and C-termini, and some flexible loop regions. These domains were individually subjected to MR calculations. Using this strategy, we obtained a clear solution, only when we used a model composed of the N-terminal 39–125 amino acids, combined with a model encompassing the C-terminal amino acids 128–168 and 178–196, in MR calculations. MR calculations confirmed that two MAM_{K201A} molecules are present in the asymmetric unit.

In the final refined model, two MAM_{K201A} mutant molecules form a dimer in the asymmetric unit cell (Fig. 1a). This MAM_{K201A} structure in apo form is apparently different from that of MAM_{wt} in receptor-bound forms (see below).^{5; 7} These differences were clearly verified by the high quality of the electron density maps as shown in Fig. 1b. For the apo MAM_{L50A} and apo MAM_{wt} structures, 10 dimers similar to that of the apo MAM_{K201A}, but different from the MAM_{wt} structure in receptor-bound forms,^{5; 7} are present in the asymmetric unit cell (Supplemental Fig. 1a,b). In each of these structures, residues 1–16 of monomer 1 and residues 1–18 of monomer 2, within the homodimer, are disordered.

The MAM K201A mutant displays a domain-swapped dimer

Upon completion of the model construction, it became obvious that the C-terminal domain (CTD) of the apo K201A mutant adopts a conformation very different from that of the receptor-bound MAM_{wt} in its co-crystal structures of the MAM-pMHC binary complex and of the MAM-pMHC-TCR ternary complex.^{5; 7} Although strikingly and unexpectedly, the two monomers of the MAM_{K201A} homodimer have exchanged more than half of the residues in their CTDs. Apparently, the two molecules are intimately intertwined, resulting in a highly twisted “knot” at the center of the elongate dimer (Fig. 1a). The central hinge region (aa. 154–156) between the two polypeptide chains is clearly defined in the electron density maps (Fig. 1b). The N-terminal domains (NTDs) of the two monomers are located at opposite sides of the central knot so that they do not contact one another at all.

The MAM_{K201A} structure is α -helical in character, and is thus similar to that of the receptor-bound MAM_{wt} determined previously.^{5; 7} The interface within the MAM_{K201A} homodimer is mainly formed by the CTDs of the monomers. In addition, a portion of helix $\alpha 2$ (aa 61–67), and the loop (aa 67–71) connecting helices $\alpha 2$ and $\alpha 3$, of the NTD of each monomer are involved in contacts for dimerization. Helix $\alpha 5$, which is the first helix of the CTD, folds back toward the N-terminal 4-helical bundle, as was seen for the receptor-bound MAM_{wt}^{5; 7} (see below). After a connecting loop that ends at residue 155, the polypeptide chain forms the helix $\alpha 6$ that, instead of folding back, extends out from its own domain and meets the cognate domain of the other monomer within the dimer to continue the rest of the fold (Fig. 1a and b).

The MAM_{K201A} monomer structure in the apo form does not represent a stable fold, as it exposes many hydrophobic residues, which are only shielded from the solvent by the interactions involved in dimer formation. The dimer interface buries a total of 6,662 Å² of solvent-accessible surface (SAS) area with an equal contribution from each of the two monomers. The buried SAS area (3,331 Å²) of each MAM monomer represents about 25% of the total surface area of 13,734 Å² for a MAM molecule. Nearly 75% of residues in the CTD participate in both hydrophobic and hydrophilic interactions at the dimer interface (Supplemental Table 1). Three residues (Trp178, Trp179, and Tyr194) each contribute more than 100 Å² buried surface area, upon dimer formation. In addition, as many as 30 hydrogen

bonds are formed at the interface (Supplemental Table 1). Among the hydrogen bonds formed across the dimer interface, only six are present in the receptor-bound monomer form (Supplemental Table 1).^{5; 7}

The MAM_{K201A} dimer in the crystal retains its dimerization status in solution

We observed a MAM_{K201A} 3D-domain swapped dimer in the crystals. However, we previously found that only a small fraction of the MAM molecules exist as homodimers in solution, even at very high protein concentration.¹³ This raises a question whether there is an equilibrium between the monomer and dimer of MAM in solution. We performed gel filtration analyses of the MAM_{K201A} molecule in crystals and in solution (Fig. 1c). Our results indicated that the majority of the MAM_{K201A} molecules rescued by dissolving the MAM_{K201A} crystals retain as dimers, with a relative small portion as monomers (Fig. 1c, black). In contrast, the MAM_{K201A} molecules rescued from several clear crystallization drops (incubated with the same crystallization reagents for the same incubation time, but no crystals formed) displays mainly a monomer peak, with a small fraction as dimers (Fig. 1c, green). The MAM_{K201A} molecules without incubation or incubated with crystallization reagents for a shorter period of time (2 hrs) show similar size distribution as the ones from clear drop, with a much smaller fraction in monomer form (Fig. 1c, cyan and red). Our results indicated that there is a monomer-dimer equilibrium for the MAM molecules in solution.

The 3D domain-swapped dimer is preserved in the structures of the apo MAM_{wt} and apo MAM_{L50A}

The 3D domain-swapping of the MAM_{K201A} molecules is unexpected, raising the question of whether the domain-swapped dimer is present in MAM_{wt}. Therefore, we determined the crystal structures of the apo MAM_{wt} and apo MAM_{L50A}, of which the crystals are isomorphous, but differ from that of MAM_{K201A} (Table 1).¹² Structure solutions for MAM_{wt} and MAM_{L50A} could be obtained only when the apo MAM_{K201A} dimer structure was used as a search model in MR calculations. No solution could be generated using the MAM_{wt} structures^{5; 7} in receptor-bound form as search models.

The MR results suggested that the MAM_{wt} and MAM_{L50A} structures in apo form are similar to that of the 3D domain-swapped MAM_{K201A} dimer, but not to that of the receptor-bound MAM_{wt}.^{5; 7} Subsequent refinements confirmed this conclusion. Although we only performed limited steps of structural refinements due to the low resolutions for the diffraction data of the MAM_{wt} and MAM_{L50A} crystals, the result can be interpreted with confidence in light of known high-resolution structure of MAM_{K201A}. In addition, due to the existence of multiple copies (10 copies) of dimers that can be used in non-crystallographic symmetry averaging refinement, the electron density maps appeared to be of much higher quality than would be expected for the low resolutions (data not shown).

In both MAM_{wt} and MAM_{L50A} crystals, 20 molecules pair one another to form 10 copies of 3D domain-swapped dimer, resulting in two fiber-like structures (Supplemental Fig. 1). Each fiber is composed of five dimers rotating through a 5₁-like skew axis (Supplemental Fig. 1a). In addition, we noted that apo MAM_{wt} could also be crystallized in a crystal morphology (diamond shape) similar to that for apo MAM_{K201A} after microseeding with the MAM_{K201A} crystals.¹² Although we did not evaluate those diamond-shaped MAM_{wt} crystals due to their small sizes (compared to the plate-shaped crystals for MAM_{L50A} and MAM_{wt} as we analyzed here), it is reasonable to believe that they were isomorphous to the cubic MAM_{K201A} crystals described here. Nevertheless, our results indicated that the 3D

domain-swapped dimer as seen in the apo MAM_{K201A} mutant is essentially the same as the apo MAM_{wt} structure.

The MAM K201A and L50A mutations do not affect the binding of MAM to its receptors

Although it has been shown that a small proportion of MAM_{wt}, MAM_{L50A}, and MAM_{K201A} molecules can form homodimer at high concentration,¹³ it was still unexpected to find a dimer of the MAM molecules in the 3D domain-swapped form. This raised a question of whether the MAM_{K201A} and MAM_{L50A} mutant are biologically active. To resolve this issue, we first performed studies of MAM_{K201A} binding to both soluble TCR and peptide-MHC (pMHC) molecules, using analytical ultracentrifugation (AUC) sedimentation equilibrium (Fig. 2).

Using a reversible (A+B $\rightleftharpoons$ AB) hetero-association model with a global analysis of the equilibrium data in SEDPHAT,¹⁵ we estimated a dissociation constant (K_D) of 104 nM, with an root-mean-square deviation (rmsd) of 0.006, for MAM_{K201A} interaction with class II MHC HLA-DR1/HA (Fig. 2a). Previously, using a I¹²⁵-labeled cellular assay,¹⁶ we measured the K_D values, ranging from 25 nM to 59 nM, for MAM_{wt} binding to class II MHC molecules expressed on human B cells or on HLA-DR1 transfected cells. A K_D of 104 nM is higher than the values from the cellular assay; however, the discrepancy could result from differences in the methods used. Indeed, we recently determined a binding affinity of 100 nM for MAM_{wt} and HLA-DR1/HA interaction, using the same AUC method.¹⁷ Clearly, the binding affinity of MAM_{K201A} for soluble HLA-DR1/HA is nearly identical to the more recent value for MAM_{wt}, indicating that the K201A mutation has no effect on the MAM-pMHC interactions. This conclusion is consistent with our co-crystal structural data for the MAM/HLA-DR1/HA and TCR/MAM/HLA-DR1/HA complexes that we determined previously.^{5, 7} In the structures of the complexes, Lys201 is located in the MAM CTD, whereas HLA-DR1/HA only interacts with the MAM NTD.⁵

Since we also determined the crystal structure of the MAM_{L50A} mutant which showed similar 3D domain-swapped dimer, we measured the affinity of MAM_{L50A} binding to pMHC. Similarly, a K_D of 69 nM was estimated for the MAM_{L50A} binding to HLA-DR1/HA complex (Fig. 2b); this value is somewhat lower than that for MAM_{wt}. Nevertheless, the conclusion that the L50A mutation does not significantly affect MAM binding to class II pMHC is again consistent with our co-crystal structural data.⁵ Although Leu50 is in the MAM NTD, it is not involved in interaction with HLA-DR1/HA.⁵ Thus neither the K201A nor the L50A mutation affects MAM binding to class II MHC HLA-DR1/HA.

Although the two mutations do not affect pMHC binding, they could affect TCR binding. Therefore, we determined the dissociation constants of these mutants and MAM_{wt} to TCR using the AUC sedimentation equilibrium (Fig. 2c-e). With a protocol similar to that used in analyzing the MAM/pMHC interactions, the K_D values were estimated to be 140 nM, 180 nM, and 140 nM, for the binding of a soluble single-chain TCR D10 to MAM_{wt}, MAM_{K201A}, and MAM_{L50A}, respectively. Apparently, the K201A and L50A mutations do not alter the binding affinity between MAM and the D10 TCR. Consistently, neither K201 nor K50 interacts with the TCR 2C in the pMHC-MAM-TCR ternary complex structure that we determined previously.⁷

The MAM_{K201A} and MAM_{L50A} mutants are biologically active

To further investigate whether these mutations affect the biological activity of MAM, we examined IL-2 production by murine T cell hybridomas expressing various V β elements, in

response to the presentation of MAM_{wt} and MAM_{mut} by human Epstein-Barr virus-transformed B lymphoblastoid cells LG2 (DR1/DR1). As shown in Figure 2f-g, when the LG2 cells were used as antigen-presenting cells, the MAM mutants K201A and L50A were both able to stimulate the activation of T cell clones 3DT (Vβ8.2+) and Kmls15.8 (Vβ15.8+), two MAM-responder murine T cell clones.^{16; 18} Comparable results were obtained when another MAM-reactive T cell line, RG17 (Vβ6+), was used (data not shown). In addition, our results showed that MAM_{wt} and mutants stimulated the activation of 3DT T cells in a dose-dependent manner. In contrast, no dose-dependent effect was observed for the activation of Kmls15.8 T cells by these molecules. It is possible that the activation of kmls15.8 T cells had already peaked, even at 0.25 ng/ml concentration, the lowest tested for the MAM proteins. We noticed that the IL-2 production is nearly 4 times higher for kmls15.8 cells than it is for 3DT T cells stimulated by the MAM proteins. A lower concentration of MAM_{wt} or mutants may be required to bring the reaction into the dose-dependent range, for the kmls15.8 T cells. Nevertheless, our results demonstrated that the K201A and L50A mutants both stimulated the activation of T cells as efficiently as did MAM_{wt}, indicating that the mutants are biological active.

Comparison of structures of apo MAM_{K201A} and receptor-bound MAM_{wt}

Previously, we determined the crystal structure of the MAM-pMHC binary complex and the crystal structure of a complete pMHC-MAM-TCR ternary complex at high resolutions, in both of which the MAM_{wt} displays very similar monomer structures.^{5; 7} Therefore, we can compare the structure of apo-MAM obtained here with that of MAM_{wt} complexed with its receptors (here we used MAM_{wt} in the MAM-pMHC complex for comparison). We also noticed that the structure of apo MAM_{wt} is similar to that of MAM_{K201A} and that MAM_{K201A} is as biologically active as MAM_{wt}. Therefore, we would use the apo MAM_{K201A} structure, due to its high resolution, to perform all of the following structural comparisons with the receptor-bound MAM_{wt} structure.^{5; 7} However, the conclusions should be generally extensible to MAM_{wt}.

When MAM_{wt} is complexed with receptors,^{5; 7} the receptor-bound MAM_{wt} molecule is in a monomer form, showing a two domain structure with 10 α-helices. The NTD (aa 1–125) is composed of a four-α-helical bundle, while six helices are in the CTD (aa 126–213) (Fig. 3a). In contrast, only nine helices are present in the monomer of the 3D domain-swapped structure of the apo MAM_{K201A} (Fig. 1a and 3b). Superposition of the NTDs of the receptor-bound MAM_{wt}^{5; 7} onto the counterparts of the MAM_{K201A} monomer and dimer indicated that the N-terminal four-helical bundle is conserved in the 3D domain-swapped structure of MAM (Fig. 3c,d). Indeed, structural superposition of the NTDs of MAM_{K201A} and receptor-bound MAM_{wt} resulted in a small rmsd of 0.58Å, indicating an overall similarity.

Although MAM_{K201A} forms a 3D domain-swapped dimer, a “closed” monomer, according to the nomenclature of Eisenberg,⁹ could be “reconstituted” from the domain-swapped dimer, in such a way that the “reconstituted” MAM_{K201A} monomer comprises residues 1–155 of monomer 1 and residues 156–212 of monomer 2 (Fig. 3e). The broken point between residues 155 and 156 is the hinge region for the 3D domain-swap. Clearly, the CTD of the MAM_{K201A} monomer “reconstituted” from the 3D-domain swapped dimer is dissimilar to that of receptor-bound MAM_{wt} (Fig. 3, panels e-g), in terms of positions and rearrangement of secondary structure elements and the overall domain orientations. When the N-terminal four-helical bundles of two MAM structures (residues 1–125) are optimally superimposed, the CTD of the “reconstituted” MAM_{K201A} monomer (residues 126–213) is required to be rotated about 36° to achieve the best alignment with the corresponding domain of MAM_{wt} in receptor-bound form (Fig. 3, panels f-g). The overall rmsd for the alignment of the CTDs is

significantly larger than that for the alignment of NTDs (data not shown) (Fig. 3g), indicating a dissimilarity. As shown in Fig. 3g, upon the best alignment of the CTDs, helices $\alpha 5$, $\alpha 9$, and $\alpha 10$ are superimposed very well. In contrast, the position of helix $\alpha 6$, which is directly downstream of the domain-swap hinge region, is changed completely. As a consequence, helix $\alpha 7$ in the receptor-bound MAM form joins with helix $\alpha 8$, to become one long α -helix ($\alpha 7'$) in the domain-swapped form (Fig. 3g). The new helix $\alpha 7'$ in the “reconstituted” MAM_{K201A} are at a position similar as the original helix $\alpha 8$ in the receptor-bound MAM form, when the CTDs are best superimposed (Fig. 3g).

The N-terminal loop of MAM is flexible, implying that conformational changes could occur upon receptor binding

In the structures of MAM_{wt} and MAM_{L50A}, five 3D domain-swapped dimers pack through a 5₁-like skew axis to form a fiber-like oligomer (Supplemental Fig. 1). The fiber-like oligomer is formed through the molecular interactions between the N-terminal four-helical bundles of the domain-swapped dimers. Supplemental Fig. 2a shows a close-up view of representative configurations of two dimers within the fiber-like oligomer. Interestingly, within the unit cell of the MAM_{K201A} crystal, four MAM_{K201A} dimers can form a similar fiber-like oligomer through crystallographic symmetry (Supplemental Fig. 1c,d). The molecular interactions between the MAM_{K201A} oligomers are also mediated by similar four-helical bundle-bundle contacts, but with a 4₁- instead of 5₁-like skew axis (Supplemental Fig. 1c,d). Whether the oligomerization of MAM molecules in the crystals has any biological implications is currently unknown. However, 3D domain-swapped dimerization and oligomerization have been posited to participate in disease-related aggregation such as Alzheimer's disease.^{9; 10; 11}

The fiber-like oligomer gives an alternative dimer (other than the domain-swapped dimer) for the MAM molecules (i.e. the red and purple molecules in Supplemental Figure 2a to form one alternative dimer). When we used the NTDs to superimpose the receptor-bound MAM_{wt} structure to the two molecules in the “alternative” MAM dimer, we found that the N-terminal loops of MAM_{wt} in the receptor-bound form would need to change their conformation, if steric clashes between the “alternative” dimer were to be avoided (Supplemental Fig. 2b). Indeed, in the structures of apo MAM_{K201A}, MAM_{L50A}, and MAM_{wt}, the majority of the N-terminal loops composed of residues 1–25 are disordered, with only residues 17–25 well defined in the electron density maps. As shown in Supplemental Figure 2b, it is obvious that the conformations of residues 17–21 of this partially ordered loop (red and purple colored) in the “alternative” apo MAM dimer (red and purple) are different from the conformations of the counterparts of MAM_{wt} (green and magenta) in the receptor-bound form. It has been proposed that the N-terminal loop of MAM_{wt} plays significant roles in recognition by the receptor, class II MHC molecule HLA-DR1/HA complex.⁵ In the MAM-HLA-DR1/HA and TCR-MAM-HLA-DR1/HA complexes,^{5; 7} the N-terminus of MAM had a nearly identical structure, composed of a well-ordered long loop of 25 residues (1–25), which wraps around the α -helical bundle in a fashion nearly perpendicular to the bundle's long axis. In the complex structures, this loop directly interacts with the antigenic peptide loaded into the pMHC peptide-binding groove.^{5; 7} In contrast, the loop composing residues 17–21 of the MAM molecule in the domain-swapped “alternative” dimer displays an extended conformation, running nearly parallel to the axis of the helical bundle. Currently, we do not know the conformation of the rest of N-terminal residues beyond residue 17 in these apo MAM 3D domain-swapped structures, due to disorders. However, we expect their conformation to differ from the conformation of this loop in the receptor-bound form, given the potential for steric clashes within the “alternative” dimer interface, as noted above (Supplemental Fig. 2b). Our results indicate

that the N-terminal loop of MAM molecule is flexible, and thus receptor binding is likely to involve conformational changes in the N-terminal loop.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The apo MAM_{K201A}

(a) A 3D domain-swapped MAM_{K201A} dimer in the asymmetric unit. The two MAM_{K201A} monomers are colored yellow and red. Nine helices of one monomer are labeled. Three phosphate groups are shown in ball-and-stick representation. The N- and C-termini are labeled.

(b) A representative $(2F_o - F_c)$ electron density map, contoured at 1.0σ , at the dimer interface of the MAM_{K201A} mutant structure. The orientations of the molecules are very similar to the ones in panel (a). The two MAM_{K201A} molecules are again colored yellow and red, with respective pink and cyan electron density maps. Here and in the following figures (unless otherwise specified), atoms are colored as follows: carbon (mol. 1), yellow; carbon (mol. 2), pink; nitrogen, blue; oxygen, red; phosphate, green. Residues in each monomer are labeled.

(c) Gel filtration analyses of MAM_{K201A}. The line colors of gel filtration profiles for samples and standard are as follows: Blue, Bio-Rad molecular weight (MW) standard (MW

labeled); Black, MAM_{K201A} rescued by dissolving crystals of MAM_{K201A} in 100 µl running buffer; Green, MAM_{K201A} resolved from clear drops from the MAM_{K201A} crystallization trials in 100 µl running buffer; Red, 2 µl of MAM_{K201A} at 5.6 mg/ml briefly incubated with 2 µl of crystallization reagents for 2 hours; Cyan, MAM_{K201A} alone without any treatment. All samples were run on a small Superdex-200 column (GE HealthCare) using an AKTA Purifier, with a buffer comprising 0.1 M potassium sodium phosphate pH 6.2, 0.2 M NaCl. The buffer is similar to that for crystallization.

Figure 2. Functional analyses of interactions of the MAM mutants with their receptors
 (a-e) Sedimentation equilibrium (SE) studies were conducted at a temperature of 20°C and at three rotor speeds (20K (□) rpm, 25K rpm (○), and 30K rpm (△)). The HLA-DR1/HA complex was prepared using a refolding protocol as described.^{5; 19; 20} Soluble single-chain TCR D10 (Vβ8.2Vα2) was prepared using a refolding protocol as described.²¹ Protein mixtures (100 μl), at various concentrations in a buffer containing 150 mM NaCl, 10 mM HEPES, pH7.4, 1 mM EDTA, were loaded into the Epon double-sector centerpieces. Reference cells were loaded with 110 μl of reference solutions. All equilibrium absorbance

profiles were acquired at 280-nm wavelength. The equilibrium sedimentation data were analyzed with the software SEDPHAT (<http://www.analyticalultracentrifugation.com>).²² Data analysis was performed by global least-squares analysis of data from multiple concentrations and multiple rotor speeds, using conservation of mass constraints.²² Panels a-e show the representative absorbance distributions for the sedimentation equilibrium of (a) 11.4 μM MAM_{K201A} and 2.4 μM HLA-DR1/HA complex, (b) for MAM_{L50A} (15 μM) and HLA-DR1/HA complex (1 μM), (c) for MAM_{wt} (8.3 μM) and scD10 (6.6 μM), (d) for MAM_{K201A} (7.6 μM) and scD10 (7.6 μM), and (e) for MAM_{L50A} (9 μM) and scD10 (8 μM).

(f-g) T cell proliferation assay of MAM_{wt}, and the MAM_{K201A} and MAM_{L50A} mutants using LG2 cells as antigen-presenting cells. The biological functions of MAM_{wt} and mutants were assessed in an IL-2 dependent CTLL assay, as previously described.¹⁶ Briefly, human Epstein-Barr virus-transformed B lymphoblastoid cells LG2 (10×10^4 cells/well) were co-cultured in triplet with T cells (80×10^4 cells/well) clones 3DT (V β 8.2), RG17 (V β 6) or Kmls15.8 (V β 15.8) in a 96-well plate, and were then stimulated with various concentration of MAM_{wt}, MAM_{K201A}, and/or MAM_{L50A}. Controls were cultured similarly, but were not stimulated with the MAM proteins. After 24 h of incubation at 37°C, 100 μl of the supernatant were added to 1×10^4 IL-2-dependent CTLL-2 cells in a 100 μl volume. [³H]Thymidine was then added (0.5 μCi /well). Cells were harvested after 16h, and [³H]thymidine incorporation was measured on a Coulter β -counter.

(f) Dose-dependent IL-2 responses of 3DT (V β 8.2) T cells stimulated by MAM_{wt}, MAM_{K201A}, and MAM_{L50A}.

(g) IL-2 responses of Kmls15.8 (V β 15.8) T cells to stimulation of concentration series of MAM_{wt}, MAM_{K201A}, and MAM_{L50A}.

Figure 3.

Structural comparison of the 3D domain-swapped apo MAM_{K201A} to MAM_{wt} in its receptor-bound monomer (unswapped) form.⁵ Panels (a) and (b) show individual receptor-bound MAM_{wt} (unswapped) monomer in crystal structure of the MAM/HLA-DR1/HA complex⁵ and the apo MAM_{K201A} molecule in the 3D domain-swapped dimer (this manuscript), respectively. Panels (c) and (d) show structure superimposition of the NTDs of receptor-bound MAM_{wt} monomer with the counterpart of the apo MAM_{K201A} 3D-swapped dimer (panel c) or one apo MAM_{K201A} monomer of the 3D-swapped dimer (panel d), respectively. Panels (e) to (g) show structural comparisons of the CTD of the receptor-bound MAM_{wt} with the counterpart of the “reconstituted” MAM_{K201A} monomer as defined in the manuscript.

(a) Ribbon representation of the MAM_{wt} (unswapped) monomer in crystal structure of the MAM/HLA-DR1/HA complex.⁵ The N- and C-termini are labeled in panels (a) and (b).

(b) Ribbon representation of the apo MAM_{K201A} molecule in the 3D domain-swapped dimer.

(c) Structure superimposition of MAM_{wt} (green) in receptor-bound (unswapped) form⁵ with the apo MAM_{K201A} 3D domain-swapped dimer (red and yellow). The N-terminal four-helical bundle of the receptor-bound MAM_{wt} (green)⁵ was superimposed to the counterpart of one of the apo MAM_{K201A} molecules (red) in the domain-swapped dimer (also for panels (d) and (e)). An overall similarity of the N-terminal helical bundle was observed.

(d) Structure superimposition of MAM_{wt} (green)⁵ in receptor-bound form with one of the apo MAM_{K201A} molecules in the 3D domain-swapped dimer (red). Compared to panel (c), the yellow apo MAM_{K201A} molecule was removed, in order to highlight the overall dissimilarity of the CTDs between the apo MAM_{K201A} and MAM_{wt} in receptor-bound form.

(e) Structure superimposition of MAM_{wt} (green) in receptor-bound form with the “reconstituted” MAM_{K201A} monomer composed of residues 17–155 from one monomer (red) and residues 156–213 from the other monomer (yellow) in the 3D domain-swapped MAM_{K201A} dimer. The residue numbers at the hinge region are labeled. The NTD and CTD are marked.

(f) Comparison of relative orientations of the CTDs of MAM_{wt} (green) in receptor-bound form and of the “reconstituted” MAM_{K201A} monomer (red and yellow), as shown in an orientation different from that in panel (e), upon the best alignment of the molecule’s NTDs.

(g) After best alignment of the NTDs, re-superposition of the MAM_{wt} CTDs (green) in receptor-bound form onto the “reconstituted” MAM_{K201A} monomer (36° rotation of the MAM_{K201A} CTD is required) (red and yellow). Helices α6, α7 and α8 from MAM_{wt} in its receptor-bound form (unswapped) are labeled green; helices α6 and α7’ from the “reconstituted” MAM_{K201A} are labeled yellow. The well-aligned helices α5, α9, and α10

(according to the numbering of the structure of the (unswapped) receptor-bound MAM_{wt}) are labeled black.⁵

Table 1

Diffraction data collection and structure refinement statistics

Crystal	MAM _{K201A}	MAM _{L50A}	MAM _{wt}
Data collection			
Space group	<i>P</i> 4 ₁ 32	<i>P</i> 2 ₁	<i>P</i> 2 ₁
Cell parameters	<i>a</i> = <i>b</i> = <i>c</i> =181.9 Å	<i>a</i> =163.8 Å <i>b</i> =93.0 Å <i>c</i> =210.9 Å β =93.7°	<i>a</i> =164.4 Å <i>b</i> =93.0 Å <i>c</i> =211.5 Å β =93.5°
Resolution (Å)	2.8 (2.9–2.8) ^a	3.6 (3.7–3.6)	4.0 (4.1–4.0)
Redundancy	6.9 (5.4)	3.5 (3.2)	1.7 (1.6)
Completeness (%)	95.9 (96.8)	97 (91.5)	83.1 (87.3)
Average <i>I</i> / σ (<i>I</i>)	9.6 (2.7)	16 (4.7)	3.6 (1.6)
<i>R</i> _{sym} (%)	11.7 (69.2)	10.0 (35.8)	15.0 (41.8)
Refinement			
Resolution range (Å)	100–2.8	Structure solutions were obtained, and only partially refined.	
No. of Reflections	24853		
<i>R</i> _{free} (%)	28.7		
<i>R</i> _{work} (%)	22.9		
No. of molecules/asym. unit	2	20	20
No. of atoms			
Protein	3259		
PO ₄ ³⁻	3		
Water	34		
Ave. B-factors (Å ²)	69.2		
Protein	75.1		
PO ₄ ³⁻	84.4		
Water	61.5		
R.m.s. deviations			
Bond length (Å)	0.008		
Bond angle (°)	1.4		
Ramachandran analysis:	Residues in		
Most favored	85.7%		
Additional allowed	11.6%		
Generously allowed	2.7%		
Disallowed region	0%		

^aValues in parentheses are those for the highest-resolution shell.

Soluble MAM_{wt} and the K201A and L50A mutants were expressed as GST-fusion proteins, as described previously.^{5; 12} Crystals were grown at room temperature in hanging drops, by mixing of 2 μ l of protein solution with an equal volume of reservoir solution containing 13–15% PEG 3350, 0.2 M NaCl, 5% ethanol glycol, 5% glycerol, and 0.1 M potassium sodium phosphate, pH 6.2. The crystals of MAM_{wt} and MAM_{L50A} belong to space group *P*2₁. Two forms of the MAM_{K201A} crystals were obtained, with one form isomorphous to the MAM_{wt} and MAM_{L50A} crystals, and one in a different form with space group *P*4₁32.¹² Prior to data collection, all crystals were transferred to a cryo-protectant solution (mother

liquor supplemented with 25% glycerol), and were flash-cooled in a nitrogen-gas stream. Diffraction data for the MAM L50A and K201A mutant crystals were collected at 100K at Beamline X4A of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL). After extensive crystal screens, a set of diffraction data for MAM_{WT} was collected to 4.0 Å resolution using an in-house Rigaku Micromax-007 X-ray facility and a RaxisIV++ detector at the Wadsworth Center. Data were processed, scaled, and reduced using the programs HKL2000,²³ d*TREK,²⁴ and the CCP4 suite.²⁵ The structures of the crystals of these MAM molecules were determined by molecular replacement (MR) with the program PHASER.¹⁴ Further manual model building was continued with TURBO FRODO²⁶ and CNS1.0²⁷ and PHENIX²⁸ were used for model refinement.