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Cutting Edge: Evidence for a Dynamically Driven T Cell Signaling Mechanism

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T cells use the αβ TCR to bind antigenic peptides presented by MHC proteins (pMHC) on APCs. Formation of a TCR–pMHC complex initiates T cell signaling via a poorly understood process, potentially involving changes in oligomeric state, altered interactions with CD3 subunits, and mechanical stress. These mechanisms could be facilitated by binding-induced changes in the TCR, but the nature and extent of such alterations are unclear. Using hydrogen/deuterium exchange, we demonstrate that ligation globally rigidifies the TCR, which, via entropic and packing effects, will promote associations with neighboring proteins and enhance the stability of existing complexes. TCR regions implicated in lateral associations and signaling are particularly affected. Computational modeling demonstrated a high degree of dynamic coupling between the TCR constant and variable domains that is dampened upon ligation. These results raise the possibility that TCR triggering could involve a dynamically driven, allosteric mechanism. The Journal of Immunology, 2012, 188: 5819–5823.

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Abbreviations used in this article: ANM, anisotropic network model; HDX-MS, hydrogen/deuterium exchange monitored by proteolysis and mass spectrometry; β2m, β2-microglobulin; pMHC, peptide/MHC complex.

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Materials and Methods

Protein expression and purification

Recombinant A6, DMF5, and HLA-A2 were refolded from inclusion bodies and purified as described previously (12). Constructs were stabilized either with an engineered disulfide bond (13) or a C-terminal leucine zipper (14). Synthetic peptide was purchased from Genescript.

Hydrogen/deuterium exchange

Exchange was initiated by diluting samples at 25°C in 25 mM HEPES, 50 mM NaCl, pH 7.4, 10-fold with the same buffer made with 99.9% \( ^2 \text{H}_2\text{O} \). For analysis of free A6, DMF5, and Tax/HLA-A2, protein concentration after dilution was 10 µM. For analysis of bound A6, concentrations after dilution were 10 µM TCR, 40 µM pMHC, resulting in 94% occupancy. These concentrations were reversed for analysis of bound Tax/HLA-A2. For examination of high affinity DMF5, concentrations after dilution were 20 µM TCR and 80 µM pMHC, resulting in 100% occupancy. For A6 with nonconjugate MART-1 26-35/HLA-A2, concentrations after dilution were 10 µM TCR and 45 µM pMHC.

After dilution into \( ^2 \text{H}_2\text{O} \), 5-µl aliquots were removed at time points ranging from 5 s to 10 h. For each aliquot, exchange was quenched at 0°C with 100 µl 0.1% trifluoroacetic acid at pH 2.4, conditions under which the rate of hydrogen/deuterium exchange is minimal (15). Pepsin (8 µl at 1 mg/ml) was added and digestion performed for 5 min before freezing in liquid nitrogen. Samples were stored at ~80°C until analysis.

Identification of peptic fragments of TCR and HLA-A2

Peptic fragments were identified by LC/MS/MS. Five hundred nanograms of the pepsin digest was loaded onto a 75 µm × 15 cm C12 column. Peptides were eluted using an Eksigent Nano UltraLC into the nanospray source of an AB Sciex 5500 QTrap spectrometer. An individual survey scan was performed, and up to six dependent MS/MS acquisitions were collected every cycle. Data were searched using the Paragon algorithm in thorough search mode with no enzyme specificity (16). The sequences of the TCR, HLA-A2, \( \beta_2 \)-microglobulin (\( \beta_2m \)), and potential contaminants (keratins, pepsin, and the Escherichia coli genome) were searched. False detection rates were determined using a decoy-database strategy (17). In all cases, the relevant proteins were the top hit and all peptides passed on for further analysis identified at a 99% confidence interval. An example LC/MS/MS spectrum is shown in Supplemental Fig. 1.

MALDI mass spectrometry

A matrix solution of 2,5-dihydroxybenzoic acid in 50:50 0.1% trifluoroacetic acid (pH 2.4) and acetonitrile was prepared and mixed 1:1 with the acidified protein on a chilled MALDI target. The target was dried under vacuum to minimize back exchange; experiments in which fully deuterated fragments were processed the same way indicated back exchange never exceeded 5%. Spectra were acquired on a Bruker Autoflex III Smartbeam MALDI-TOF mass spectrometer. The instrument was calibrated with a standard peptide mixture prior to collection of each data set.

MALDI data processing

The centroid (mass weighted average) of the isotopic envelope for each peptide was calculated via a spreadsheet after data export. The level of backbone deuterium incorporation for each peptide was calculated as:

\[
\text{% deuteration} = 100 \times \left( \frac{m_n}{m_{100}} - \frac{m_{99}}{m_{100}} \right)
\]

where \( m_n \) is the centroid mass, \( m_{99} \) is the mass of the undeuterated sample, and \( m_{100} \) is the mass of the peptide in which the backbone is fully deuterated. In all cases examined, exchange was complete by 10 min as shown in Fig. 1B and Supplemental Fig. 1; thus, percent deuteration at 10 min was used for comparisons.

Surface plasmon resonance

Surface plasmon resonance experiments with the wild-type and high-affinity variant of DMF5 were performed using a steady-state assay with a Biacore 3000 instrument as previously described (12). Solution conditions were 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20, pH 7.4, 25°C.

Anisotropic network model calculations

Anisotropic network model (ANM) calculations were performed using the ANM server with a cutoff for interactions between \( \alpha \) carbon atoms of 15 Å and a distance weight for interactions between \( \alpha \) carbon atoms of 2.5 (18). Calculations were performed on the separately solved structures of A6 and its complex with pMHC (14, 19).

Structure-based computational design of a high-affinity DMF5 variant

Design of the high-affinity DMF5 variant was performed using the ZAFFI algorithm (20) and the structure of wild-type DMF5 in complex with the decameric MART-1 epitope (21). Briefly, mutations were simulated with the interface protocol of Rosetta 2.3 (22), using gradient-based minimization to refine backbone and torsion angles and rigid-body position before and after mutation (this was superior for reproducing binding affinities compared with the fixed backbone protocol used originally). From this analysis, the threshold of the ZAFFI filter function was raised to 0.3. Computational mutagenesis of 31 DMF5 residues resulted in 589 mutants. Ten of the highest scoring mutants were evaluated experimentally, and two that led to enhanced binding (\( \alpha \text{D26Y, B1L98W} \)) combined to generate the high-affinity variant.

Results and Discussion

Ligation by pMHC globally rigidifies the A6 TCR

Although NMR remains the most powerful method for investigating protein motions, complications resulting from the size, complexity, and stability of TCRs and their complexes have limited NMR studies of TCR–pMHC interactions, requiring the use of simplified systems (23). To circumvent these challenges, we used hydrogen/deuterium exchange monitored by proteolysis and mass spectrometry (HDX-MS) as a tool to probe flexibility (reviewed in Ref. 24). In HDX-MS, protein prepared in aqueous solution is incubated in \( ^2 \text{H}_2\text{O} \), digested into fragments, and backbone deuteron incorporation for each fragment determined via mass spectrometry. Increased levels of deuteron incorporation indicate that backbone amides become more accessible to exchange through greater sampling of more open, solvent-exposed states, whereas lower levels of incorporation indicate that amides are protected from exchange through less frequent excursions into solvent-exposed states. HDX-MS has been used in a number of cases to explore protein motions and their changes and has been particularly useful in characterizing low-frequency motions in samples not easily adaptable to NMR (24).

We began by performing HDX-MS on the ectodomain of the well-characterized A6 TCR in the absence and presence of its cognate pMHC ligand, the Tax peptide presented by the class I MHC HLA-A*0201 (HLA-A2). The A6 TCR is particularly amenable to this analysis, as its high affinity toward Tax/HLA-A2 allows full receptor occupancy to be achieved without requiring concentrations of pMHC that would hinder detection of TCR signals in the analysis of the exchange reactions (12). As shown in Fig. 1A, the A6 TCR showed decreased levels of deuteration upon ligation. Data for each peptide fragment were collected as a function of time out to several hours, but in all cases examined, exchange was essentially complete within 10 min as shown in Fig. 1B and Supplemental Fig. 1. The average deuteration of the free TCR after 10 min of exchange was 35%, whereas in the complex the average deuteration after 10 min of exchange was 18% (Fig. 1C). These values were reproducible, with a repeat experiment yielding free and bound levels of 34% and 16%, respectively. As shown in Fig. 1C, the reduced deuteration was distributed across the TCR, indicating that TCR ligation results in a global reduction in structural fluctuations, extending from the variable to the constant domains, although the extent varies throughout the molecule. Similar global reductions in flexibility upon ligand binding have
been seen in other protein systems, such as the allosterically modulated DNA binding protein BirA (25).

As expected, peptide fragments covering the A6 CDR loops showed reduced deuteration. Beyond the CDR loops, reduced deuteration was also seen for other key regions of the TCR, including a more than 3-fold reduction in deuteration for a fragment spanning the β-chain FG loop, a prominent structural feature that forms part of a cavity that has been proposed as a docking site for CD3γ (26). A molecular dynamics simulation has shown that the CD3γ subunit also rigidifies upon binding (in this case an mAb) (27). It is not known if CD3γ likewise rigidifies upon ligand binding; however, stronger binding of one or both of the CD3e subunits to the TCR resulting from pMHC-induced rigidification could play a role in T cell triggering.

Although the FG loop is the most prominent feature affected in the TCR constant domains, the reduction in flexibility includes other elements also implicated in either TCR oligomerization or interactions with CD3 subunits (3, 28). This includes the α-chain AB loop, which has been suggested to play a role in mediating interactions between adjacent TCRs on T cell membranes. Reduced AB loop flexibility could underlie a prior observation of a conformational change occurring upon TCR ligation (4), which is observed to a small extent when comparing the bound and free structures of A6 (19).

The ectodomain of the A6 TCR used for the data in Fig. 1 included an engineered disulfide bond near the base of the TCR constant domains to ensure heterodimer stability and facilitate proper chain pairing during protein refolding (13). An experiment with an alternate construct stabilized instead by a C-terminal heterodimeric coiled coil (14) also showed reduced deuteration upon binding at regions distant from the Ag binding site (Supplemental Fig. 1), indicating that the results in Fig. 1 are not a consequence of the method used to stabilize the receptor. A control experiment in which HDX-MS was performed with A6 before and after mixing with noncognate ligand did not show reduced deuteration (Supplemental Fig. 1).

**Ligation by pMHC globally rigidifies the DMF5 TCR**

To ask if the reduction in exchange occurring upon binding was general or specific to A6, we repeated the HDX-MS experiments using the DMF5 TCR, which recognizes the MART-1,266–35 peptide presented by HLA-A2 (21). However, DMF5 binds more weakly than A6, and achieving high receptor occupancy required excess ligand concentrations that negatively affected detection of TCR signals by MALDI mass spectrometry. We thus used a high-affinity DMF5 variant, produced using the recently developed structure-guided TCR design algorithm ZAFFI (20). Surface plasmon resonance analysis of 10 DMF5 point mutants predicted to improve affinity identified two that when combined yielded a variant that bound with an affinity of 43 nM, more than 250-fold tighter than wild-type (Fig. 2A).

HDX-MS experiments with the high-affinity DMF5 variant showed a reduction in hydrogen/deuterium exchange upon ligation that closely resembled that observed with A6 (Fig. 2B, Supplemental Fig. 1). The average deuteration of DMF5 before and after binding was 37% and 19%, respectively, with a repeat experiment yielding values of 35% and 18%. As with A6, the distribution was reduced across the TCR and included the FG loop and the AB loop, as well as

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Hydrogen/deuterium exchange reveals that the A6 TCR undergoes a global reduction in flexibility upon pMHC binding. (A) MALDI-TOF mass spectra for the fragment of the FG loop (sequence WTQDRAKPVTQ of A6 free (blue) and bound to Tax/HLA-A2 (red)) 10 min after initiation of hydrogen/deuterium exchange. (B) Time courses for the exchange reaction of the FG loop fragment in (A) demonstrates that in both free and bound A6, exchange follows simple kinetics and is complete within 10 min. (C) Percent deuteration of each fragment of free and ligand-bound A6 at the 10-min time point. Fragments including the CDR and the AB, DE, and FG loops in the constant domains are indicated. The dashed blue and red lines indicate the average deuteration of the free and bound protein, respectively.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** The DMF5 TCR also undergoes a global reduction in flexibility upon pMHC binding. (A) Steady-state surface plasmon resonance experiment showing the affinities of the native (left) and high-affinity (right) variant of DMF5 for the MART-1,266–35/HLA-A2 ligand. The high-affinity variant shows a >250-fold enhancement in binding affinity. (B) Percent deuteration of fragments of free and ligand-bound high-affinity DMF5 variant at the 10-min time point. Fragments including the CDR, AB, CC', and FG loops and the C and F strands are indicated. The dashed blue and red lines indicate the average deuteration of the free and bound protein, respectively.
other regions implicated in TCR oligomerization or association with CD3 subunits (3, 28). The reduction in exchange behavior for both DMF5 and A6 mapped to the structures of the TCRs is shown in Fig. 3.

**Highly correlated internal dynamics of the TCR are dampened upon ligation**

To investigate changes in TCR dynamics upon pMHC binding further, we performed ANM dynamics simulations, which report on low-frequency protein motions captured by HDX-MS (reviewed in Ref. 29). In ANM simulations, slow motions inaccessible to traditional molecular dynamics simulations are sampled by modeling protein structures as connected nodes whose motions are regulated by empirically determined force constants. ANM simulations have been used to study the dynamics of complex systems as large as ribosomes and have been particularly useful in exploring how ligand-induced changes in flexibility can give rise to allosteric effects. Several comparisons have shown good agreement between ANM data and experimental assessments of low-frequency protein motions, including data from hydrogen/deuterium exchange and NMR (29).

ANM simulations of the free and complexed A6 TCR reproduced the general features of the HDX-MS analysis, showing the global dampening of dynamics associated with ligand binding (Fig. 4A). The motions of the binding loops were extensively affected, as were those of the FG loop and other elements of the constant domains. We next performed a cross-correlation analysis of the data to examine the dynamic coupling between regions of the TCR. In its free state, a high degree of local, fragment-level correlated motion was observed throughout the TCR, including both within and across the Vα, Vβ, Cα, and Cβ domains (Fig. 4B). These correlated local motions were significantly reduced in the ligated state. Rather than correlated motion occurring between individual elements such as the CDR and constant domains loops, in the bound state the domains themselves moved in correlated, en bloc motions (Fig. 4C). This switch from fragment-level correlated to domain-level correlated motion suggests the TCR is mechanically optimized to respond dynamically to pMHC ligation. The interconnected nature of the Ig fold could underlie this behavior. Interdomain dynamics and their reduction upon binding could be communicated through the domain linkers, which in other modular proteins have been shown to be key elements in propagating signals from one domain to another (30). Another contributor could be the inherent flexibility in the relative positioning of the TCR variable domains (31), the reduction of which upon binding could impact the motions within and between the constant domains.

**TCR ligation also rigidifies the HLA-A2 H chain and β2m**

To determine if TCR ligation alters the dynamics of the MHC, we performed HDX-MS on Tax/HLA-A2. TCR ligation also rigidified the HLA-A2 H chain from 27 to 16% (Supplemental Fig. 2, with supporting mass spectrometry data in Supplemental Fig. 1). As expected, the α1 and α2 helices had decreased incorporation of deuterons when ligated to the TCR. However, distant regions (including sites that contact the CD8 coreceptor) also had lower levels of deuteration, indicating that, similar to the TCR, the MHC also rigidifies upon binding. β2m also rigidified, showing a reduction in deuteration from 24 to

![FIGURE 4. ANM simulations indicate the TCR possesses a high degree of correlated internal motion that is reduced upon ligand binding.](http://www.jimmunol.org/)

![FIGURE 3. Percent change in deuteration upon binding of each A6 and DMF5 fragment mapped to the TCR structure. The distributed reduction in exchange behavior stemming from the Ag binding site to the constant domains is clearly visible. As indicated by the scale, warmer colors indicate a large change, and cooler colors indicate a smaller change. Fragments colored gray were not resolved in the experiments.](http://www.jimmunol.org/)
The authors have no financial conflicts of interest.

References

Supplemental Fig. 1. Supporting mass spectrometry data. (A) MALDI-TOF mass spectra of the peptic fragment spanning the A6 CDR3α loop free and in the presence of excess Tax/HLA-A2. (B) Time course of the H/D exchange in panel A. (C) MALDI-TOF mass spectra of the peptic fragment of the FG loop of the A6 TCR stabilized via a C-terminal coiled coil free and bound to Tax/HLA-A2 after 10 minutes of exchange. The shift demonstrates that the rigifidification of the molecule is independent of the method utilized to stabilize the ectodomain. (D) MALDI-TOF mass spectra of the peptic fragment of the FG loop of the A6 TCR free and in the presence of excess of the non-cognate MART-126-35/HLA-A2 pMHC after 10 minutes of exchange. Lack of a shift indicates the changes in deuteration seen with cognate ligand are due to specific antigen recognition. (E) MALDI-TOF mass spectra of the peptic fragment of the FG loop of the DMF5 TCR free and in the presence of excess MART-126-35/HLA-A2 ligand. (F) Time course of the H/D exchange in panel E. (G) MALDI-TOF mass spectra of the peptic fragment of the α2 helix from Tax/HLA-A2 free and bound to the A6 TCR. (H) Time course of the exchange in panel G. (I) MALDI-TOF mass spectra of the peptic fragment from the region of the α3 domain that contacts CD8 in Tax/HLA-A2 free and bound to the A6 TCR. (J) Time course of the H/D exchange in panel I. (L) Sequences of the fragments in panels A - J. (K) MS/MS spectrum of the peptic fragment of the A6 FG loop. The z=2 charge state was fragmented at 665.35 m/z. Matching y-type ions are shown in red, matching b-type ions are shown in magenta. Confidence for this peptide match was >99.9% by the Paragon score.
Supplemental Figure 2. The HLA-A2 protein, including regions across the heavy chain and β2-microglobulin, rigidifies upon TCR binding. (A) Percent deuteration of peptic fragments of the HLA-A2 heavy chain and β2-microglobulin free and bound to the A6 TCR at the 10 minute time point. Fragments spanning the heavy chain α1 and α2 helices as well as regions which contact CD8 are indicated. The dashed orange and green lines indicate the average deuteration of the free and bound protein, respectively. (B) Percent change in deuteration of the HLA-A2 heavy chain mapped to the three dimensional structure. As indicated by the scale, warmer colors indicate a large change and cooler colors indicate a smaller change. Fragments colored grey were not resolved in the experiments. For clarity, the image on the left shows data only for the HLA-A2 heavy chain, whereas the image on the right shows data only for β2-microglobulin.