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Systematic mutation and thermodynamic analysis of central tyrosine pairs in polyspecific NKG2D receptor interactions

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ABSTRACT

The homodimeric, activating natural killer cell receptor NKG2D interacts with multiple monomeric ligands polyspecifically, yet without central conformational flexibility. Crystal structures of multiple NKG2D–ligand interactions have identified the NKG2D tyrosine pair Tyr 152 and Tyr 199 as forming multiple specific but diverse interactions with MICA and related proteins. Here we systematically altered each tyrosine to tryptophan, phenylalanine, isoleucine, leucine, valine, serine, and alanine to measure the effect of mutation on affinity and thermodynamics for binding a range of similar ligands: MICA, the higher-affinity ligand MICB, and MICdesign, a high-affinity version of MICA that shares all NKG2D contact residues with MICA. Affinity and residue size were related: tryptophan could often substitute for tyrosine without loss of affinity; loss of the tyrosine hydroxyl through mutation to phenylalanine was tolerated more at position 152 than 199; and the smallest residues coincide with lowest affinities in general. NKG2D mutant van't Hoff binding thermodynamics generally show that substitution of other residues for tyrosine causes a moderate positive or flat van't Hoff slope consistent with moderate loss of binding enthalpy. One set of NKG2D mutations caused MICA to adopt a positive van't Hoff slope corresponding to absorption of heat, and another set caused MICB to adopt a negative slope of greater heat release than wild-type. MICdesign shared one example of the first set with MICA and one of the second set with MICB. When the NKG2D mutation affinities were arranged according to change in nonpolar surface area and compared to results from specific antibody–antigen and protein–peptide interactions, it was found that hydrophobic surface loss in NKG2D reduced binding affinity less than reported in the other contexts. The hydrophobic effect at the center of the NKG2D binding appears more similar to that at the periphery of an antibody–antigen binding site than at its center. Therefore the polyspecific NKG2D binding site is more tolerant of structural alteration in general than either an antibody–antigen or protein–peptide binding site, and this tolerance may adapt NKG2D to a broad range of protein surfaces with micromolar affinity.

1. Introduction

Immune function for T cells, B cells, and natural killer cells often requires cross-reactivity in which one receptor must engage a range of similar protein ligands (Wucherpfennig et al., 2007), with the precise term "polyspecific" preferred to the general term "promiscuous" (Christopher Garcia et al., 2009). Polyspecific interactions have been explained by the combination of accessibility and hydrophobicity for antibody Fc domain–protein interactions, (DeLano et al., 2000) by thermodynamic rather than structural plasticity for gp130–cytokine interactions, (Boulanger et al., 2003) by rigid adaptation of hot-spots for NKG2D–ligand interactions (McFarland and Strong, 2003), and by conserved pairwise interaction motifs for T cell receptor-major histocompatibility complex interactions (Feng et al., 2007; Scott-Browne et al., 2009). None of these models of polyspecific binding requires extensive structural rearrangement. Rather than conformational alterations, these polyspecific interfaces depend on tyrosine (McFarland et al., 2003; Feng et al., 2007; Scott-Browne et al., 2009), methionine (DeLano et al., 2000), arginine, and tryptophan hot-spots (Boulanger et al., 2003), all of which are residues with polar and non-polar segments that can adopt multiple rotamers while backbones remain rigid.

Tyrosine is prominent at polyspecific interfaces. A study of polyspecific multibinding interfaces found that they are most

Abbreviations: CDR, complementarity-determining region; HEL, hen egg white lysozyme; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry.

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enriched in tyrosine (Tyagi et al., 2009). Antigen–contacting residues in the complementarity-determining regions (CDRs) of antibodies are most often tyrosine (Padlan, 1990). An automated method found tyrosine to be the most prevalent residue in antibody CDRs, which may be cross-reactive, but not in antibody epitopes, which have no requirement for cross-reactivity (Ofran et al., 2008).

Structurally, tyrosine has been called the "most versatile" of amino acids (Kossiakoff and Koide, 2008; Koide and Sidhu, 2009) due to its ability to form multiple aromatic (Chakrabarti and Bhattacharyya, 2007), hydrophobic, and hydrogen-bonding interactions. These properties allow it to fulfill diverse structural and thermodynamic roles, even within a single interface. For example, three tyrosines have different roles in the nuclear cap binding complex binding its ligand (Worch et al., 2009); tyrosine anchors drive coupled folding and binding of an intrinsically disordered protein to its partner (Espinoza-Fonseca, 2009); and two tyrosines pin a loop that plays an important role in the structure–entropy relationship for the protein Tsg101 binding a peptide ligand (Killian et al., 2009). Protein interfaces with restricted chemical diversity are dominated by tyrosine in multiple roles (Fellouse et al., 2004, 2006; Koide et al., 2007). Minimalist interfaces with the highest Tyr content are the most specific (Birtalan et al., 2008). When the amino acid diversity of a minimalist Tyr-and-Ser interface is expanded, the newly introduced residues play a supporting role and conformationally optimize Tyr contacts (Gilbreth et al., 2008).

The role of tyrosine in binding of the antibody HyHEL-63 to hen egg white lysozyme (HEL) has been studied extensively, locating five antibody Tyrs and one HEL Tyr at the interface that raise ΔG of interaction by more than 1 kcal/mol when mutated to alanine (Li et al., 2002). By quantifying the binding thermodynamics of a series of mutations, the magnitude of the hydrophobic effect was found to be doubled at a central site (Li et al., 2005) relative to a peripheral site (Sundberg et al., 2000). A similar series of mutations at the C-terminal residue of a peptide that binds a pocket on the protein T-Mod also found a doubled hydrophobic effect (Jackrel et al., 2009).

The NK cell lectin-like receptor NKG2D is a homodimeric, polyspecific protein that forms at least six different crystallographically defined half-site interfaces with its inducible, monomeric protein ligands (Strong and McFarland, 2004). Successful engagement with these ligands drives NK response in cancer immunity (Guerra et al., 2008) and autoimmunity (Van Belle, 2009). NKG2D–ligand interfaces are structurally diverse, are dominated by neither hydrophobic nor electrostatic interactions (McFarland et al., 2003), and show a flat van't Hoff slope consistent with an interaction thermodynamically characterized by moderate enthalpy, releasing a few kcal/mol of heat to the surroundings (McFarland et al., 2003). Computational alanine scanning identified two central hot-spot tyrosines (Tyr 152 and Tyr 199) in each half-site as the most energetically prominent ligand-binding NKG2D residues (McFarland et al., 2003). Tyr 152 has also been specifically implicated in the interactions of NKG2D with heparin and sulfate-containing polysaccharides (Higai et al., 2009).

NKG2D must be polyspecific because it engages multiple ligands in each organism (e.g., MICA and MICB in humans), in contrast to specific antibody–antigen interfaces that have undergone affinity maturation for a single surface. We hypothesized that this polyspecificity may lead to structural and thermodynamic differences in how NKG2D uses its tyrosine residues to interact with multiple ligands. We developed a focused range of structural alterations to the NKG2D tyrosines and measured binding affinity and thermodynamics with three different protein ligands. The results from NKG2D–ligand interactions compare the hydrophobic effect at this interface to the hydrophobic effects observed for HyHEL-63 tyrosine mutants interacting with HEL and T-mod interacting with peptide tyrosine mutants.

2. Materials and methods

2.1. Mutagenesis and protein production

NKG2D mutants were produced by site-directed mutagenesis using the Quikchange II kit (Stratagene). The extracellular domains of NKG2D and its ligands were produced as described for MICA (Li et al., 2001), MICB (Holmes et al., 2002), and NKG2D and MICdesign (MICN69W_K152E_K154D) (Lengyel et al., 2007). Briefly, recombinant gene expression was induced in BL21-DE3 cells, producing inclusion bodies that were washed with detergent and solubilized in 8 M urea, then refolded by stepwise dialysis in more dilute urea solutions. Refolded NKG2D was purified by ion-exchange chromatography, while MIC proteins were purified by nickel–NTA chromatography due to a C-terminal 6xHis tag engineered into the crystal construct. The resulting proteins were purified by size-exclusion chromatography and >95% purity was confirmed with SDS-PAGE gels. Protein concentration was determined by BCA assay (Thermo Scientific) and/or 280 nm absorbance from a Nanodrop UV–Vis spectrometer. The two methods of protein concentration determination agreed to within 10%.

2.2. Protein binding analysis

MIC ligands in 10 mM acetate buffers (pH 5.0 or 5.5) were attached to CM5 research-grade sensor chips at low ligand densities ($R_{max} < \sim 500$) using the BIAcore amine coupling kit (GE Healthcare Life Sciences) on a BIAcore 3000 instrument for surface plasmon resonance (SPR) analysis. The surface was exposed to serial dilutions of mutant NKG2D at the specified temperature for different periods depending on time to equilibrium: for 90 s at a flow rate of 40 μL/min (for wild-type and Ile, Val, and Leu mutants) or for 4 min at 25 μL/min (for Trp, Phe, Ser, and Ala mutants, which exhibited slow kinetics at some temperatures; Supplementary Fig. 1). Average equilibrium response was plotted against NKG2D concentration, then fit to a hyperbola representing binding using BIAEvaluation software version 3.2. Data were measured in triplicate or quadruplicate series and averaged at either 5 temperatures or 9 temperatures; for lines with 9 temperatures, the additional temperature points did not affect van't Hoff enthalpies beyond reducing the reported error, consistent with Monte Carlo simulations of thermodynamics (Zhukov and Karlsson, 2007). (MICdesign binding wild-type NKG2D did not come to equilibrium for temperatures colder than 25 °C, so those points were not used in its equilibrium van't Hoff plot.)

Wild-type NKG2D and wild-type MICA equilibrium binding correlate with kinetic binding measurements and with binding as measured by size-exclusion chromatography assay; the numbers reported here for equilibrium thermodynamic measurements agree within error with thermodynamics determined by kinetics (Lengyel et al., 2007). Thermodynamics of MICB binding wild-type NKG2D measured at equilibrium were previously reported (McFarland et al., 2003). Thermodynamics of MICdesign binding wild-type NKG2D measured using kinetics and confirmed with isothermal titration calorimetry (ITC) were previously reported, confirming that the interaction transfers heat to its surroundings under constant pressure and temperature, and therefore enthalpy of interaction is negative (Lengyel et al., 2007). The correspondence of SPR and ITC enthalpies has been noted previously in other experiments (Horn et al., 2001; Day et al., 2002).

2.3. Computational analysis of NKG2D mutation

Structural models of the effect of NKG2D mutation on the complex were built from the NKG2D-MICA complex structure (PDB ID