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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.

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Keywords

natural killer cell receptors; mutagenesis; polyspecific protein-protein interactions; killer lectin-like receptors; van't Hoff thermodynamics

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, Allen et al. 2007), with the precise term “polyspecific” preferred to the general term “promiscuous” (Christopher Garcia, Adams et al. 2009). Polyspecific interactions have been explained by the combination of accessibility and hydrophobicity for antibody Fc domain-protein interactions, (DeLano, Ultsch et al. 2000) by thermodynamic rather than structural plasticity for gp130-cytokine interactions, (Boulanger, Bankovich 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, Bond et al. 2007; Scott-Browne, White 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 and Strong 2003; Feng, Bond et al. 2007; Scott-Browne, White et al. 2009), methionine (DeLano, Ultsch et al. 2000), arginine, and tryptophan hot-spots (Boulanger, Bankovich 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 enriched in tyrosine (Tyagi, Shoemaker 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 (Ofra, Schlessinger 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, Jankowska-Anyszka 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, Kravitz et al. 2009). Protein interfaces with restricted chemical diversity are dominated by tyrosine in multiple roles (Fellouse, Wiesmann et al. 2004; Fellouse, Barthelemy et al. 2006; Koide, Tereshko et al. 2007). Minimalist interfaces with the highest Tyr content are the most specific (Birtalan, Zhang 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, Esaki 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, Urrutia 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, Huang et al. 2005) relative to a peripheral site (Sundberg, Urrutia 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, Valverde 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, Tan et al. 2008) and autoimmunity (Van Belle and von Herrath 2009). NKG2D-ligand interfaces are structurally diverse, are dominated by neither hydrophobic nor electrostatic interactions (McFarland, Kortemme 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 and Strong 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, Kortemme et al. 2003). Tyr 152 has also been specifically implicated in the interactions of NKG2D with heparin and sulfate-containing polysaccharides (Higai, Imaizumi 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, Morris et al. 2001), MICB (Holmes, Li et al. 2002), and NKG2D and MICdesign (MICN69W_K152E_K154D) (Lengyel, Willis et al. 2007). Briefly, recombinant gene expression was induced in BL21-DE3 cells, producing inclusion bodies that were washed with detergent and solubilized in 8M 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 280nm 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 seconds

at a flow rate of 40 $\mu\text{L}/\text{min}$ (for wild-type and Ile, Val, and Leu mutants) or for 4 minutes at 25 $\mu\text{L}/\text{min}$ (for Trp, Phe, Ser, and Ala mutants, which exhibited slow kinetics at some temperatures; Supp. 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, Willis et al. 2007). Thermodynamics of MICB binding wild-type NKG2D measured at equilibrium were previously reported (McFarland and Strong 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, Willis et al. 2007). The correspondence of SPR and ITC enthalpies has been noted previously in other experiments (Horn, Russell et al. 2001; Day, Baird 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 1HYR) using the module RosettaDesign within the Rosetta 2.0 suite (Kortemme, Joachimiak et al. 2004). RosettaDesign was run 100 times on each NKG2D mutant made in the A half-site, the B half-site, and both half-sites together, for both the NKG2D homodimer without MICA and for NKG2D in complex with MICA. Residues were allowed to adopt different rotamers but the backbone coordinates were fixed. Energies tended to converge for the 100 RosettaDesign runs so that the 5–10 lowest-energy runs were identical. NACCESS v2.1.1 (Hubbard 1996) with a 1.4-Å probe calculated polar, non-polar, and total solvent-accessible surface area for a representative run for bound and free proteins.

3. Results

3.1 Binding affinity of NKG2D tyrosine mutants

In a construct of human NKG2D corresponding to the extracellular domain, seven other amino acids were substituted for Tyr 152 or Tyr 199: Trp to investigate how shape and hydrogen bond position affect affinity, Ser to maintain and reposition the Tyr hydroxyl, and Phe, Ile, Leu, Val, and Ala to test the results of differently sized hydrophobic residues. Because NKG2D is homodimeric, each variant is a double mutant containing symmetric mutations in both half-sites. Fourteen recombinant mutants were produced in *E.coli* and purified by ion-exchange and size-exclusion chromatography.

We tested binding of the NKG2D mutants to three MIC ligands with similar surfaces using surface plasmon resonance. A crystal structure defines the complex of MICA with NKG2D (Li, Morris et al. 2001). (Fig. 1) In a previous study, we designed MICdesign, a MICA variant with three mutations at non-interfacial residues (N69W, K152E, and K154D), which binds more tightly than wild-type and with a faster on-rate. (Lengyel, Willis et al. 2007) Wild-type MICB is 84% identical to MICA and differs from MICA at six interface contacts

(Holmes, Li et al. 2002). MICB forms a more stable complex with NKG2D that is faster in both on-rate and off-rate (McFarland and Strong 2003). Kinetics were not measurable for some low-affinity mutants, so NKG2D binding response was determined at equilibrium to the NKG2D ligands MICA, MICB, and MICdesign at different temperatures to construct van't Hoff plots for thermodynamic analysis. (Table 1, Supp. Fig. 1)

In general, loss of two central tyrosines impaired binding by 1–4 kcal/mol (Fig. 2), in the range of affinity changes caused by mutation of a single tyrosine at other protein interfaces such as antibody-antigen (Li, Urrutia et al. 2002), T-MOD/peptide (Jackrel, Valverde et al. 2009), colicin DNase-immunity protein (Li, Keeble et al. 2004), and BLIP-TEM-1 (Wang, Zhang et al. 2007). MICA, the lowest-affinity ligand, was affected least by mutation (Fig. 2A), while the higher-affinity ligands MICdesign and MICB lost about 1–2 kcal/mol of binding energy upon mutation (Fig. 2B and 2C). The most significant difference between MICdesign and MICB is that MICdesign was more tolerant of serine substitution at both the 152 and 199 positions ($\Delta G = -5.8 \pm 0.4$ kcal/mol for Y152S binding MICdesign compared to -4.4 ± 0.1 kcal/mol for binding MICB), although in general serine mutations reduced affinity as much as or more than alanine mutations.

The structural similarities of Tyr and Trp appear to allow functional substitution despite the differences in shape and hydrogen bonding potential. Comparing position 152 to 199 shows that both tyrosines are important but not necessarily crucial to NKG2D binding, because several mutations to other large residues produce affinities near wild-type. Tyr-to-Trp mutations result in stronger binding at position 199 than at position 152 for all three ligands. In three out of six Trp mutants, tryptophan substitution is tolerated without loss of affinity: at both positions with MICA, and at position 199 with MICdesign.

Mutating position 199 to a smaller residue is consistently detrimental to forming the NKG2D-ligand interface (with the exceptions of Y199L and Y199V binding MICA). That greater loss of affinity is observed for Ala substitution at position 199 than position 152 is consistent with the relative predictions of computational alanine scanning (McFarland, Kortemme et al. 2003). For the NKG2D-MICdesign and – MICB interactions, making position 152 smaller is generally less detrimental than the same mutation at position 199, but it is only detrimental in two out of six smaller mutations for the MICA interaction, as Y152F and Y152I increase NKG2D affinity, and Y152A does not affect affinity. This unusual result for alanine mutation at position 152 may be compared to binding of the Phe mutant. Removal of the hydroxyl by Y152F mutation stabilizes MICA binding by about 1 kcal/mol, while at Y199F it destabilizes the interaction by about 1 kcal/mol. The net effect of Phe-to-Ala mutation at each site is similar. Other details show that a smaller residue shape that fits at one position may not fit at the other. At the MICA interface in particular, Leu is tolerated at both positions, but Ile is tolerated instead of Val at position 152, and Val is tolerated instead of Ile at position 199.

3.2 Binding thermodynamics of NKG2D tyrosine mutants

Van't Hoff plots constructed from linear fits of the natural log of equilibrium dissociation constants at multiple temperatures show different patterns for the three ligands (Fig. 3). This indicates that for some mutants, thermodynamics vary with mutation (Table 1). Van't Hoff enthalpies derived from the slopes are accurate to within 1–3 kcal/mol according to the reported errors. The thermodynamics of NKG2D-ligand interactions have been described as “driven by” enthalpy and/or entropy, but the concept of two opposed thermodynamic “driving forces” is problematic because all spontaneous interactions are driven by energy dispersal and therefore are entropy driven (Craig 2005). We consider that the fundamental finding of thermodynamics is simply whether heat is released to the surroundings (previously termed “enthalpy-driven”) or absorbed by the system (previously termed

“entropy-driven”). According to van’t Hoff plots, wild-type NKG2D binds MICA and MICdesign with release of a moderate amount of heat (2–4 kcal/mol) to the surroundings, while MICB releases little to no heat. Mutant van’t Hoff plots were used to determine if changes in heat transfer to surroundings accompanied changes in protein-protein interaction affinities.

For the NKG2D-MICA interaction, mutating the central tyrosines tends to reduce the heat released and therefore the enthalpy of interaction, especially for Y152A, Y199W and Y199F, resulting in steeper positive slopes on the van’t Hoff plots (Fig. 3A). For MICA, the slope of the Y199A plot is also increased relative to other van’t Hoff plots although errors are too high for thermodynamic quantitation due to low affinity. The NKG2D Y199A-MICdesign van’t Hoff plot also shows an increased slope corresponding to less heat release to the surroundings (Fig. 3B), while the NKG2D Y199A-MICB plot is flat (Fig. 3C). NKG2D tyrosine mutations also tend to reduce heat release from the wild-type MICdesign interaction, with the exception of Y152F, for which more heat is released. The NKG2D Y152F-MICB interaction shows a similar decreased van’t Hoff slope associated with additional heat transfer and enthalpy of interaction. Y152V and Y199V NKG2D mutants binding MICB show a similar decreased slope (Fig 3C). All other NKG2D tyrosine mutants bind to MICB with near-zero releases of heat, like wild-type NKG2D binding MICB. Altogether, one set of NKG2D Tyr mutants (Y152A, Y199W, Y199F, and Y199A) release less heat or absorb heat when binding MICA, indicating significantly reduced enthalpy of interaction. A different set of mutants (Y152F, Y152V, and Y199V) release more heat when binding MICB, indicating significantly increased enthalpy of interaction. MICdesign shares one reduced-heat-release mutation with MICA and one increased-heat-release mutation with MICB.

3.3 The apparent hydrophobic effect is not enhanced in NKG2D

Rosetta was used to build fixed-backbone models of the mutated NKG2D-MICA interfaces, and NACCESS calculated polar and non-polar surface areas for estimation of changes in solvent-accessible surface area changes upon tyrosine mutation at each half-site (Supp. Fig. 2). Solvent-accessible surface area changes more upon mutation at position 152 than position 199, and more for half-site B than half-site A. The range of interface area buried upon binding is similar to that seen in tyrosine-rich minimalist protein interfaces (Koide and Sidhu 2009). Compared to the HyHEL-63 tyrosine mutants interacting with HEL (Li, Huang et al. 2005), position 199 mutants bury less surface area than the specific antibody, due to the bulky side chains Met184 and Lys197 on either side (Fig. 1), and position 152 mutants bury more.

The magnitude of the hydrophobic effect can be estimated from a plot of change in binding free energy vs. change in solvent-accessible surface relative to the Y152F and Y199F mutants (Fig. 4). NKG2D mutants involving loss of surface area at the central tyrosines bind MICA more tightly relative to the phenylalanine mutants than similar mutations in a specific antibody-antigen interaction (Li, Huang et al. 2005) and a designed peptide-protein interaction (Jackrel, Valverde et al. 2009). The relationship of affinity to surface area for NKG2D is non-linear, and it may be influenced by other structural effects than hydrophobicity (e.g., homodimer flexibility not modeled by Rosetta). The relationship is also complicated by the unusual behavior of position 199 upon mutation in that Y199F and Y199A reduce affinity but Y199L and Y199V do not. The non-linearity in the data does not affect the observation that all points but one (Y199A) are located significantly below the linear relationships established for the previous two experiments in which a magnified hydrophobic effect was observed. If a linear regression is made to the NKG2D data, its slope is at maximum 10–20 cal/Å², compared to about 40 cal/Å² for a specific antibody-antigen interaction (Li, Huang et al. 2005) and for a peptide-protein interaction (Jackrel, Valverde et

al. 2009). Therefore the apparent hydrophobic effect at the center of the NKG2D-MICA interface is similar to a peripheral site in a specific antibody-antigen interaction, previously found to be $\sim 20 \text{ cal}/\text{\AA}^2$ (Sundberg, Urrutia et al. 2000), but is less than that observed at the center of specific binding sites. All NKG2D-contacting residues present in MICA are also present in MICdesign, and so the buried surface area for MICA may be used as an estimate for MICdesign. With these data, MICdesign also showed a similar, weak apparent hydrophobic effect, except for Y199A (Supp. Fig. 3).

4. Discussion

In this study, we found the affinity of interaction between three protein surfaces and a panel of NKG2D proteins systematically mutated at the central tyrosine “hot-spot” residues. All three present similar interfaces to NKG2D and show similar changes in affinity upon mutation, with smallest residues correlating with lowest affinities. The higher-affinity ligands MICB and MICdesign were less tolerant to hot-spot mutation in general than the lower-affinity MICA ligand. Koide and Sidhu noted “as tryptophan shares many attributes with tyrosine, the question remains whether tryptophan may be able to replace tyrosine as the main contributor to interface energetics” in minimalist protein interfaces (Koide and Sidhu 2009). In the context of the large, flat NKG2D-ligand interface, substitution of Trp for Tyr was allowed, especially at the 199 position, so that the larger shape and structural complexity of Trp mediated multispecific binding in this case.

The small changes in structure caused by these systematic mutations generally do not cause large changes in thermodynamic heat release. Mutation to smaller residues generally causes reduction of enthalpic heat release upon mutation, probably attributable to specific lost interactions (for example, a Tyr-to-Phe hydrogen-bond deletion mutation caused loss of binding enthalpy in a protein-ligand interaction, although enthalpic heat release still dominated binding due to Van der Waals interactions (Barratt, Bingham et al. 2005)). Some NKG2D mutants showed distinctly different van’t Hoff slopes, providing a “signature” for each ligand. We do not know what interplay among structure, dynamics, and thermodynamics causes these exceptions, although the fact that part of the MICA interface folds upon binding is an unusual feature expected to alter thermodynamics. Some connections between conformational entropy and protein structure can be made with detailed study, as for calmodulin (Marlow, Dogan et al. 2010).

Some NKG2D mutants have increased van’t Hoff slopes for MICA, while others have decreased slopes for MICB, and MICdesign shares one increased-slope mutant at position 199 with MICA and one decreased-slope mutant at position 152 with MICB. MICdesign has interfacial residues identical to MICA and shares fast binding kinetics with MICB. In particular, the alterations of MICdesign (Lengyel, Willis et al. 2007) are located beneath the disordered loop observed in the MICA structure (MIC residues 150–160 (Li, Willie et al. 1999)), and position 152 directly contacts MICA loop residues 156 and 159, while position 199 is on the other side of MIC position 159, away from the loop. If the MICdesign mutations affect loop dynamics but not the rest of the surface, then this structural feature could explain why the thermodynamics of MICdesign position 199 match MICA while the thermodynamics of the loop-contacting position 152 do not. This would connect possible disordered loop dynamics with van’t Hoff thermodynamics, and suggests similarity between MICB and MICdesign loop dynamics.

NKG2D positions 152 and 199 exhibit differences in buried surface area for fixed-backbone models of mutant structures. Position 199 is more central to the binding site and is less exposed to solvent because of its flanking residues Lys 197 and Met 184. Because Y199F causes a distinct loss of affinity to all ligands, loss of the specific Tyr OH hydrogen bond is

more detrimental at position 199. In contrast, Y152F causes a gain of affinity for MICA and maintaining its bulk is important: substituting a large hydrophobic residue (whether Y152W, Y152I, or Y152L) is permitted for MICA with no loss of binding. The tyrosine at position 152 was also observed in a different rotamer when eight half-site crystal structures were compared, whereas position 199 was always observed in the same rotamer, and position 199 was predicted to be more important to binding (McFarland, Kortemme et al. 2003). These observations combine if position 199 acts as a typical “hot spot” and to its side is position 152, an adaptable hydrophobic wall that is exposed to the solvent channel below the NKG2D homodimer interface and above the ligand.

These results demonstrate the context dependence of both binding thermodynamics and hydrophobicity. The hydrophobicity in the center of the NKG2D binding site, although not a simple linear relationship, is not magnified like it is in antibody-antigen (Li, Huang et al. 2005) or protein-peptide interactions (Jackrel, Valverde et al. 2009); the slope of the line of NKG2D mutant free energy vs. buried area is closer to that of a peripheral antibody mutation than a central one. Mutation of position 152, despite large changes in buried surface area upon mutation, has less of an effect than similar mutations elsewhere. Y199A has a large deleterious impact on binding relative to the Y199F mutant, but the relatively tight binding of Y199L, Y199I and Y199V suggest that Y199A’s loss of affinity is not due to a general strengthening of the hydrophobic effect at this position. In the crystal structure, neither position 152 nor the loops on the other side of position 199 (McFarland, Kortemme et al. 2003) appear sufficient to accomplish the structural sequestration observed at other interfaces that magnify the hydrophobic effect. Because the NKG2D interaction site is so flat, adjacent rotamers may be able to adapt and residues larger than alanine or serine may be able to substitute more effectively than they can in deep pockets.

Polyspecific binding requires multiple interaction options rather than strong interactions, and adaptability may be preserved by dispersing binding energy throughout the interface so that no one interaction dominates, not even the central tyrosine positions. A strengthened hydrophobic effect would be antithetical to the purpose of polyspecific binding. The two adjacent tyrosines may also compensate for each other when one is altered or forced to form suboptimal interactions. These characteristics may allow NKG2D to adapt to mutations as well as to multiple ligand surfaces, explaining why several NKG2D mutants, such as Y199W and Y152A, do not lose as much affinity as expected even when large structural changes occur in the center of the binding sites.

The NKG2D receptor binds its ligands as if optimized, not for affinity, but for multispecificity. Its micromolar affinity for ligands is weaker than typical antibody-antigen interactions, and it does not exhibit the magnified hydrophobic effect that is associated with binding strength and specificity. What it lacks in depth of binding strength, it compensates for in breadth of binding possibility. Its central tyrosine pairs form a set of core interactions that can tolerate significant ligand diversity and mutation, forming specific interactions with many ligands to accomplish its role in immune function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this paper

CDR	complementarity-determining region
HEL	hen egg white lysozyme

SPR	surface plasmon resonance
ITC	isothermal titration calorimetry

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Fig. 1.

Crystal structure of human NKG2D interacting with MICA showing the central tyrosine pairs, PDB ID: 1HYR (Li, Morris et al. 2001). (A) Side view of the NKG2D-MICA complex (NKG2D chain A yellow, NKG2D chain B green, MICA blue). The side chains Tyr 152 and Tyr 199 are shown as sticks with transparent surfaces. (B) Close-up view of half-site A in the vicinity of the tyrosine pair. Major residues are, from left to right, Met 184, Tyr 199, Lys 197, and Tyr 152 on NKG2D and His 79, Asp 149, Arg 74, Met 75, Arg 38, and Lys 71 on MICA. (C) Close-up view of half-site B. Major residues are, from left to right, Met 184, Tyr 199, Lys 197, and Tyr 152 on NKG2D and Asp 163, Ala 159, Thr 155, and His 156 on MICA.

Fig. 2. Changes in binding free energy of NKG2D mutants relative to wild-type NKG2D for (A) MICA, (B) MICdesign, and (C) MICB.

Fig. 3. Van't Hoff plots for NKG2D mutants binding (A) MICA, (B) MICdesign, and (C) MICB. Mutants similar in thermodynamics to wild-type are shown in gray, while those with slopes significantly greater or less than wild-type are shown in black, with mutant names to the right of each line.

Fig. 4.

Correlations between free energy of binding and solvent-accessible surface area change upon binding for MICA. All points for MICA are relative to the Y199F or Y152F mutants so that the comparison is based on non-polar surface changes only. Position 199 mutants are white circles and position 152 mutants are white squares. The slope of the linear regression fit to both NKG2D positions' data is 14 cal/mol $\text{\AA}^2$. Data for the HyHEL63-HEL antibody-antigen interaction, black squares, from (Li, Huang et al. 2005). Data for the T-Mod-peptide interaction, black circles, from (Jackrel, Valverde et al. 2009).

Binding thermodynamics of NKG2D mutants to MIC ligands.

All values are in kcal/mol. SE = standard error. $-\Delta S$ is calculated by inference from ΔG (at equilibrium at 25°C) $-\Delta H$ (from van't Hoff analysis). Values that deviate most significantly from wild-type thermodynamics for that mutant NKG2D-ligand interaction are shown in bold type.