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*J Immunol* 2012; 188:6165-6174; Prepublished online 21 May 2012; doi: 10.4049/jimmunol.1102496

http://www.jimmunol.org/content/188/12/6165

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**Supplementary Material**  [http://www.jimmunol.org/content/suppl/2012/05/21/jimmunol.1102496.DC1](http://www.jimmunol.org/content/suppl/2012/05/21/jimmunol.1102496.DC1)

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Dimerization of NKp46 Receptor Is Essential for NKp46-Mediated Lysis: Characterization of the Dimerization Site by Epitope Mapping

Micha Jaron-Mendelson,*1 Rami Yossef,*1 Michael Y. Appel,* Alon Zilka,* Uzi Hadad,* Fabian Afergan,* Benyamin Rosental,* Stanislav Engel,†‡ Shlomo Nedvetzki,§ Alex Brainman,* and Angel Porgador*

NKp46 is a primary activating receptor of NK cells that is involved in lysis of target cells by NK cells. Previous studies showed that the membrane-proximal domain of NKp46 (NKp46D2) retained the binding of NKp46 to its ligands and is involved in lysis. We studied NKp46D2 by using a peptide-based epitope mapping approach and identified an NKp46D2-derived linear epitope that inhibited NKp46-mediated lysis. The epitope, designated as pep4 (aa 136–155), interacted with NKp46, and lysis by NK cells was inhibited by the presence of pep4. Through modeling and mutagenesis, we showed that pep4 could be involved in NKp46 homodimerization. R145 and D147 contribute to the function of pep4, and R145Q mutation in recombinant NKp46 reduced its binding to target cells. At the cellular level, fluorescent resonance energy transfer analysis revealed that pep4 is indeed involved in dimerization of cell membrane-associated NKp46. We suggest that the NKp46-derived pep4 site is part of the dimerization surface of NKp46 and that NKp46 dimerization contributes to NKp46-mediated lysis by NK cells.


N atural killer cells recognize target cells via the use of a diverse array of activating and inhibitory receptors, and thus a delicate balance between inhibitory and activating signals tightly regulates NK cell activation (1–5). NKp46 is a member of a group of activating receptors collectively termed natural cytotoxicity receptors (NCRs) that are expressed by NK cells. The NCRs, which include three members—NKp30, NKp44, and NKp46—are expressed almost exclusively on NK cells and are capable of mediating direct killing of tumor and virus-infected cells by NK cells (6). NKp46 is considered the most specific NK cell marker for which an orthologous protein (NCR1) has been found in mice (7, 8). The ligands recognized by NCRs are not yet fully elucidated. NKp30 recognizes the cellular ligands B7-H6, BAT3 (9, 10), the HCMV protein pp65 (11), and heparan sulfate (HS) as a co-ligand (12). In contrast, however, the cellular ligands for both NKp44 and NKp46 are largely unknown, and the ligands identified to date for these two proteins are various virus hemagglutinins (13, 14), flavivirus E-protein for NKp44 (15) and HS as a co-ligand (12, 16–18). Vimentin was reported as cellular ligand for NKp46 (19).

To identify the ligand binding site of a receptor, a peptide-based epitope mapping approach could be taken (20). This approach was used for NKp30 based on its crystal structure, which provides a clear definition of its secondary structure and surface elements; accordingly, the authors were able to map a specific part of the NKp30 receptor successfully to identify the ligand binding site (21). However, in other cases a peptide mapping approach was used to identify allosteric epitopes, defining sites that regulate the protein’s activity but are not involved directly in ligand binding. Frequently, defined allosteric epitopes are involved in receptor homodimerization as shown for IL–17R and MyD88 (22, 23). Homodimers were observed in the reported three-dimensional crystal structures of the NKp30 and NKp44 receptors (21, 24); yet, for NKp46, no homodimers were observed (25, 26).

In the current study, we investigated NKp46-domain 2 (NKp46D2), which is the membrane-proximal ligand binding domain of NKp46 (16, 27, 28). We used a peptide-based epitope mapping approach and identified an NKp46D2-derived linear epitope that manifested binding to NKp46D2 and inhibited NKp46-mediated lysis. To study further whether this epitope regulates NKp46 activity through inhibition of homodimerization, we used modeling, mutagenesis, and fluorescent resonance energy transfer (FRET) technology. We conclude that this epitope is part of the dimerization surface of NKp46 and that NKp46 dimerization contributes to NKp46-mediated lysis.

Materials and Methods

Cells

Cell lines used in this work were as follows: HeLa (human cervical adenocarcinoma, ATCC no. CCL-2), HEK293T (SV40 large TAg-transfected HEK293 cells, ATCC no. CRL-11268), PC-3 (human prostate adenocarcinoma, ATCC no. CRL-1435), 721.221 (EBV-transformed human B cells) and MIN-6 (secreting pancreatic β cell line) (29), and...
NK-92 (a human NK lymphoma, ATCC no. CRL-2407). Wild-type CHO-K1 cells and the mutant derivatives CHO pgA-745 and CHO pgD-677 were kindly supplied by Dr. Jeff Esko (University of California at San Diego, La Jolla, CA) and have been characterized in detail elsewhere (16, 30).

Abs and recombinant NK receptors

The following Abs were used: anti-NKp46 mAb (MAB1850; R&D Systems) and biotin-conjugated mouse anti-human CD107a/LAMP-1 mAb (9835-08; Southern Biotech). Allophycocyanin-conjugated F(ab’)2 goat anti-human IgG, allophycocyanin-conjugated F(ab’)2 goat anti-mouse IgG, and allophycocyanin-conjugated streptavidin were obtained from Jackson ImmunoResearch (109-136-098, 115-136-068, and 016-130-084, respectively). Generation of mouse polyclonal anti-NKp46 or naive serum was previously described (13). The production of NKp46-Ig, NKp30-Ig, NKp62D-Ig, and LIR1-Ig was described elsewhere (16, 27, 31). To generate the recombinant NKp46-R145Q mutation (accession number NP_004820.1; http://www.ncbi.nlm.nih.gov/), we used a construct coding for NKp46-T225N-Ig that binds cellular ligands similar to NKp46-Ig (28) and mutated arginine to glutamine (R145Q). In all experiments involving NKp46-R145Q-Ig, control rNKp46 was parental NKp46-T225N-Ig.

NKp46-derived peptides

Peptides were synthesized with/without biotin using PEPscreen technology, which is a peptide synthesis platform that utilizes Fmoc chemistry (Sigma-Aldrich). Peptides covered the NKp46D2 and the hinge region (aa 121–254; accession number NP_004820.1; www.ncbi.nlm.nih.gov/). Selected peptides were also ordered from BioSight (BioSight Israel). Stock solutions of peptides (2 mg/ml) were solubilized in DDW–10% DMSO and stored in frozen aliquots.

Constructs and transfections
cDNA for full-length NKp46 (accession number NM_001145457.1; www.ncbi.nlm.nih.gov) was cloned into the HindIII and BamHI sites of the pECPn1 and pEYPn1 vectors (Clontech Laboratories) to generate pNKp46-ECFP or pNKp46-EYFP fusion constructs, respectively. The NKp46 insert included an upstream Kozak consensus sequence (GCCACC), as well as an additional two bases (GC) following the start codon (GCCACC), as well as an additional two bases (GC) following the start codon. The NKp46 insert included an upstream Kozak consensus sequence (GCCACC), as well as an additional two bases (GC) following the start codon. The NKp46 insert included an upstream Kozak consensus sequence (GCCACC), as well as an additional two bases (GC) following the start codon.

Flow cytometry

Binding of rNK receptors and binding inhibition assays were performed as follows: 1 x 10^5 target cells were incubated with 2 μg of the NK receptor-Ig and 5 or 10 μg of the peptide in staining solution consisting of 0.5% (w/v) BSA and 0.05% sodium azide in PBS at 4°C for 1.5 h. Then, cells were washed and stained with allophycocyanin-conjugated anti-human IgG. Dead cells were detected with propidium iodide. Percentage of inhibition was calculated as the percent of the cell population showing a reduced staining by the NK receptor-Ig in the presence of peptides compared with reference positive staining by the NK receptor-Ig without peptides. To assess the direct binding of peptides to target cells, 1 x 10^5 tumor cells were incubated with 10 μg biotin conjugated peptides for 1 h at 4°C, washed, stained with allophycocyanin-conjugated streptavidin, and analyzed by FACS. Flow cytometry was performed using a FACSCan II or FACS Calibur (BD Biosciences), and results were analyzed using Diva 6.1.2 or CellQuest 3.3, respectively.

Isolation and culture of primary NK cells

NK cells were isolated from the peripheral blood of healthy donors using a human NK cell isolation kit (Miltenyi Biotec). NK purity was greater than 90% (CD3^- CD56^). Purified NK cells were cultured in CellGro SCGM serum-free medium (CellGenix) supplemented with 10% heat-inactivated human plasma from healthy donors, 1 mM sodium pyruvate, 2 mM L-glutamine, MEM nonessential amino acids, 1% penicillin/streptomycin, 10 mM HEPES (Life Technologies), and 300 IU/ml human IL-2 (Biological Industries Israel).

Cytotoxicity and CD107a degranulation assays for NK cell activity

The flow cytometry cytotoxicity assay was previously described (32). Effector human primary NK cells were labeled with CellTrace CFSE (C3554; Invitrogen). Analysis of 7-aminoactinomycin D for negative-CFSE target tumor cells was performed using a FACSCan II, and data were analyzed by Diva 6.1.2. For blocking experiments, NK cells were preincubated with mouse polyclonal anti-NKp46 serum or with naive serum for 30 min at 4°C before being combined with target cells. The NK degranulation assay was performed as previously described (17, 33).

Kinetic analysis by surface plasmon resonance

We used the ProteOn XPR36 protein interaction array system, NLC chip, and ProteOn Manager 2.1 version 2.10.3.8 (Bio-Rad Laboratories) to measure the affinity of rNK receptors to NKp46-derived peptides. The ligand immobilization process was performed with PBS–0.005% Tween 20 (P7949; Sigma-Aldrich) at a flow rate of 30 μl/min. Ligand binding (NKp46-derived peptides) ranged between 200 and 500 RU. Different analyte concentrations (5000–312.5 nM or 2000–125 nM, and 0 nM) of rNK were injected, each followed by regeneration of the surface using 50 mM NaOH. For kinetic measurements, data processing was done using the 1:1 Langmuir binding model.

Modeling

The structure of the D2 domain was extracted from the crystal structure of the human NKp46 receptor ectodomain [Protein Data Bank (PDB) code 1oll; www.rcsb.org]. The PatchDock server for rigid docking (34, 35) was used to generate initial docking solutions for D2 homodimers. Ten top-ranked D2 dimer structures from PatchDock were forwarded to the FireDock server (36, 37) for refinement. The first cycle of refinement used a “restricted” refinement mode that allows only the chaging residues at the dimer interface to be flexible, and the second cycle used a “full” refinement mode that allows all the interface residues to be flexible. The final refinement used mutation rigid-body optimization cycles (250) and the factor 0.95 for scaling of the atomic radii used in energy calculations to decrease the extent of acceptable steric clashes in the final refined solutions. Next, a single top-ranked solution from the FireDock was solvated in a water box using the solvate module implemented in the VMD program (38), and the whole system was submitted for minimization and equilibration at 310K using the NAMD program (39) and CHARMM (40) force field for proteins. Initial equilibration of the water molecules was carried out for 50 ps with the protein kept rigid. It was followed by a 50-ps simulation with constraints applied only to the backbone atoms of the protein. Finally, a completely relaxed system was simulated for 200 ps, sufficient time to achieve equilibration (according to the RMSD variance of the protein atoms during the trajectory). The KFC server (41) was used to analyze the protein–protein interface in the final model, and the salt bridges primitive of NAMD was used to describe salt bridges formed in the protein throughout the trajectory.

FRET

Twenty-four hours after transfection (previously described), cells were transferred and seeded at a concentration of 2.5 x 10^4 cells per well on either uncoated or anti-NKp46 precoated chambered μ-slides (80862; ibidi) and incubated overnight at 37°C. Then, cells were gently washed twice with PBS and fixed with 2% paraformaldehyde for 10 min at room temperature, washed, and FRET efficiency was assessed by confocal microscopy. Fluorescent images were acquired on a FluoView FV1000 confocal system (Olympus) using a ×63 (NA 1.35) UPLSAPO objective (Olympus). The acquired images were cropped and pseudo-colored as follows: CFP, cyan; YFP, yellow. No image enhancement procedures were performed. FRET was measured by the donor-sensitized acceptor fluorescence technique as described previously (42, 43). Briefly, three images were acquired for each set of acquisition parameters: YFP excitation/CFP emission image (YFP channel); CFP excitation/YFP emission image (CFP channel); and CFP excitation/YFP emission image (FRET channel). A set of reference images was acquired from single-labeled CFP or YFP-expressing cells for each set of acquisition parameters, and a calibration curve was derived to allow elimination of the non-FRET components from the FRET channel. The FRET efficiency was calculated on a pixel-by-pixel basis using the following equation: FRET_{corr} = FRET_{raw}/(FRET_{max} + CFP) x 100%, where FRET_{corr} is the pixel intensity in the corrected FRET image, and CFP is the intensity of the corresponding pixel in the CFP channel image.
Results

**NKp46-derived pep4 inhibits the binding of recombinant NKp46 to tumor cells**

We previously showed that ligands to NKp46 interact with the membrane-proximal domain (NKp46D2) and with the hinge region connecting NKp46D2 to the cell membrane (27, 28). In the current study, we dissected NKp46 function by dividing NKp46D2 and the hinge region into 24 overlapping, 20mer peptides with a 15-aa overlap between successive peptides. We first studied whether these 24 peptides could inhibit binding of rNKp46 to target HeLa cells. Only two peptides, peptide 4 (pep4: aa 136–155, accession number NP_004820.1; http://www.ncbi.nlm.nih.gov/) and peptide 5 (pep5: aa 141–160), inhibited binding upon coincubation with NKp46D2-Ig (Fig. 1A, shown for pep4). As NKp46-derived mock peptides shown in Fig. 1 and throughout the study, we used the two ends of the 24 overlapping peptides, peptide 1 (mock1: aa 121–140) and peptide 24 (mock2: aa 236–254). The sequences of pep4, pep5, and the two mock peptides are depicted in Fig. 1B.

NKp46-Ig binding was also inhibited by pep4 and pep5, yet to a lesser extent than the binding of NKp46D2-Ig (Fig. 1C). Peptides 4 and 5 overlap and in accordance, the 15mer-shared core sequence (Fig. 1B, boldface sequence) also inhibited NKp46-Ig binding to tumor cells (Fig. 1D). Human NKp46 recognizes ligands on human and non-human tumor cell lines (27). In accordance, pep4-mediated inhibition of rNKp46 binding was evident for numerous tumor cell lines from human (HeLa, PC-3, HEK293T, 721.221) and non-human (CHO-K1 and MIN-6) origin (Fig. 1D and data not shown). The binding inhibition was specific to NKp46, as pep4 did not induce significant binding inhibition for other NK receptor-Igs such as NKp30-Ig, NKp44-Ig, and LIR1-Ig (Fig. 1E).

**NKp46-derived pep4 inhibits NK degranulation and lysis of tumor cells**

We next investigated whether pep4 could influence NK cell function. Target HeLa cells were plated, and pep4 or mock peptide was added just before adding effecter human primary NK cells (see *Isolation and culture of primary NK cells*). Lysis of target cells was significantly suppressed when pep4 was added compared with addition of mock peptide (Fig. 2A, p < 0.01). Similarly, pep4-mediated inhibition of lysis was obtained for target 721.221 and CHO-K1 cells (Supplemental Fig. 1). To verify that pep4 inhibits NKp46-mediated lysis, anti-NKp46 serum was used to block the receptor on human primary NK cells. As above, in the presence of only control serum, pep4 inhibited lysis compared with mock binding to tumor cells (Fig. 1D).

**FIGURE 1.** Incubation with NKp46-derived pep4 and pep5 reduced binding of rNKp46 to target cells. Target cells were stained with 2 μg/well of NK receptor-Igs in the presence of 10 μg/well pep4, pep5, 15mer-shared core sequence, or mock1. The NK receptor-Ig staining intensities were determined by flow cytometry using fluorescent allophycocyanin-conjugated F(ab’)2 goat anti-human IgG. (A) HeLa cells were stained with NKp46D2-Ig in the presence of pep4 or mock1. Results are shown as histograms overlay. (B) Pep4, pep5, 15mer-shared core sequence (boldface), mock1, and mock2 sequences. Amino acid numbers are according to NKp46 accession number NP_004820.1. (C–E) Reduced staining of NK receptor-Igs mediated by the presence of peptides is shown as percent inhibition compared with staining without peptides (see *Materials and Methods*). (C) HeLa cells were stained with NKp46D2-Ig in the presence of the indicated peptides. (D) CHO-K1, HeLa, HEK293T, and PC3 cells were stained with NKp46D2-Ig in the presence of the indicated peptides. The NKp46D2-Ig binding profile for each of the cell lines is shown in the corresponding inset. (E) HeLa cells stained with NKp46D2-Ig, NKp44-Ig, NKp30-Ig, or LIR1-Ig in the presence of titrated amounts of pep4 ranging from 0.625 to 10 μg/well or 10 μg/well of mock peptides. The results (A, C–E) are from one representative experiment of at least three performed. Bars, ±SD.
peptide (Fig. 2B, \( p < 0.01 \)). The lysis of target cells was mediated by NKp46; incubation with anti-NKp46 serum and mock peptide significantly reduced lysis of target cells by effector NK cells compared with incubation with control serum and mock peptide (Fig. 2B, \( p < 0.01 \)). In contrast, when pep4 was present, no blocking effect was observed for anti-NKp46 serum or mock peptide (Fig. 2B). Inset: NKp46-expressing NK-92 cell line was preincubated with or without mouse polyclonal anti-NKp46 serum for 30 min at 4˚C before being combined with target cells at 15:1 E:T cell ratio and the indicated peptides. Percentage of specific lysis was determined by flow cytometry analysis. (C) Comparative analysis of CD107a expression on human primary NK cells coculated with HeLa or 721.221 cell lines for 4 h at the indicated E:T cell ratio in the presence of pep4 or mock2. Cells were then washed and stained for CD107a cell surface expression. The results are from one representative experiment of six (A) and three (B, C) experiments performed. Bars, \( \pm SD \). *\( p < 0.05 \), **\( p < 0.01 \).

To assess further the influence of pep4 on NK activation, we stained human primary NK cells for CD107a (LAMP-1) cell surface expression, which is a marker for NK cell degranulation and functional activity. NK cells and HeLa or 721.221 target cells were cocultured for 4 h at different E:T ratios in the presence of pep4 or mock peptide. The percentage of CD107a+ NK cells was significantly reduced when cocultured with target cells in the presence of pep4 (Fig. 2C, \( p < 0.05 \)).

**Pep4 interacts directly with NKp46 and not with target cell-expressed ligands**

To assess the mechanism through which pep4 influences NKp46-mediated NK function, we first examined whether pep4 blocks NKp46 binding to its putative ligands. Our group has published that NKp46 interacts with the HS moiety presented by HSPG as a coreceptor (26). In accordance, NKp46-Ig manifested reduced binding to CHO cells lacking HS, CHO-677, and CHO-745 compared with parental CHO-K1 cells (16). We thus tested the influence of pep4 on NKp46 binding to these cells. Pep4 suppressed to the same extent the binding of NKp46-D2-Ig to CHO-K1, CHO-677, and CHO-745 cells (Fig. 3A). Previously, we made NKp46-D2-Ig with a mutation in the HS binding site (NKp46-D2-Q4T1-Ig), which manifested reduced binding to target cells compared with HS (27). Pep4 inhibited the binding of NKp46-D2-Ig and NKp46-D2-Q4T1-Ig to the same extent (Fig. 3B). To test further whether pep4 could bind to a putative ligand of NKp46 on target tumor cells and consequently block NKp46 binding, we stained different target cells with biotinylated pep4. We could not detect pep4 binding to target tumor cells (Fig. 3C). Streptavidin-based multimeric forms of biotinylated pep4 also did not stain target cells as well (data not shown). We therefore tested whether pep4 binds to NKp46-D2 directly rather than to target cells. Pep4 displayed a characteristic binding curve to NKp46-D2 (K\( _d \) = 1.6 \( \times 10^{-7} \) M); no significant interaction could be detected for pep4 with the other NK receptors or for NKp46-derived mock peptide with NKp46-D2 (Fig. 3D). We further tested whether biotinylated pep4 can bind to the NKp46-expressing NK-92 cell line; biotinylated pep4 did not bind to these cells, but adding HS-binding peptide to the cells induced pep4 binding to the NKp46-expressing NK-92 cells (Fig. 3E). NKp46 binds HS (16); we therefore hypothesize that NK-p46-expressed HS could mask the pep4 binding site and that addition of excess HS-binding peptide displaces the NKp46-bound HS and exposes the pep4 binding site.

**Modeling of pep4 binding to NKp46**

Based on our results indicating direct interaction of NKp46-D2-derived pep4 with NKp46-D2 (Fig. 3D), we hypothesized that pep4 corresponds to a sequence contributing to NKp46 dimerization through homodimerization of NKp46D2. We therefore used molecular modeling approach to gain structural insights into the mechanism of the formation of the NKp46 dimers (see Materials and Methods). Because a flexible docking of the entire NKp46 ectodomains is impractical due to the excessive flexibility in the hinge region connecting D1 and D2, we resorted to a docking modeling of the isolated D2 domain. As a monomeric unit, we used a structure of the isolated D2 domain that we extracted from the crystal structure of the human NKp46 receptor ectodomain (PDB code 1I0L). We built our model based on the 1I0L structure rather than the 1p6f structure (26) because the latter did not allow a low-energy complex of D2 monomers to be formed using a rigid docking procedure (as described in Materials and Methods).

According to the docking model, the D2 domains of NKp46 receptor form C2 symmetrical (parallel) homodimers (Fig. 4). An
extensive dimer interface is formed by the residues 122–128, 130–133, 143–147, and 204–207 contributed by both D2 monomers (Fig. 4; amino acid numbers are according to accession no. NP_004820.1; www.ncbi.nlm.nih.gov). The complex is stabilized by shape complementarity, hydrophobic contacts distributed throughout the interface with a distinct cluster comprising the residues V128, P132, P205, and V206, and electrostatic contributions including hydrogen bonds and two salt bridges (Fig. 4). The salt bridges are positioned at the opposite sides of the interface along the dimer's symmetry axis. The first salt bridge is formed by the residues R145 and D147 contributed by the opposite D2 monomers, and the residues K207 and E133 form the second salt bridge (Fig. 4). Molecular dynamics simulation of the complex revealed that the R145/D147 and K207/E133 interdomain salt bridges were stable and persisted throughout the trajectory, although R145 was able to form an alternative to D147 contact with D122 in the opposite monomer in some frames (Fig. 4). The residues R145 and D147 are located in the middle of the pep4 sequence as well as in the 15mer-shared core sequence of pep4 and pep5 and may contribute to the formation of a pep4 epitope involved in interaction with NKp46.

**Mutagenesis of pep4 and rNKp46 to characterize amino acids involved in pep4–NKp46 interaction**

We next tested the contributions of different amino acids in pep4 to its capacity to inhibit NKp46 binding, including those amino acids (R145 and D147) that were suggested by the model to contribute to NKp46D2 dimerization through salt bridges (Fig. 4). We analyzed shorter versions of pep4 and introduced alanine substitutions of specific amino acids. In agreement with the model, mutagenesis analysis revealed that R145 and D147 are essential for pep4’s ability to inhibit NKp46D2-Ig binding, as mutation of either
amino acid eliminated the inhibition conferred by pep4 (Table I). Shortening of the 15mer-shared core sequence (Fig. 1C) to a 13mer by removing T141 and F142 also sufficed to eliminate inhibition (Table I). Yet, alanine substitution of T141 and F142 had no effect on pep4-conferred inhibition. This indicates potential importance of peptide length on its conformation. Although C144 and L146 were predicted by the model to be part of the dimer interface, their contribution through van der Waals forces is small and cumulative. Thus, substitution of C144 or L146 with alanine appears as insufficient to affect the ability of pep4 to inhibit NKp46D2-Ig binding to tumor cells. T150 and S151 were not predicted to contribute to dimerization, and alanine substitution of T150 and F153 reduced inhibition from 81 to 59%, yet the high SD renders the effect insignificant ($p > 0.05$).

To understand better the contribution of the predicted R145–D147 pairing to the homodimerization of NKp46, we studied these residues in the context of the whole NKp46 protein. R145 was a better candidate to mutate as the model predicted that D147 could be replaced by D122 for the interaction with R145. Recombinant NKp46 bearing the R145Q mutation reduced its binding to tumor cells compared with that of rNKp46; this reduction was significant as shown by the summary of three independent experiments (Fig. 5A, 5B, $p < 0.01$). In addition, the NKp46-R145Q mutation suppressed the inhibition effect of pep4; pep4-mediated inhibition was significantly higher for rNKp46 compared with that for rNKp46-R145Q (Fig. 5C, $p < 0.01$). This was also observed for the 15mer-shared core sequence of pep4 and pep5. As expected, incubation with NKp46-derived mock peptide did not inhibit binding to cells of either rNKp46 or the R145Q mutant (Fig. 5C).

**FRET analysis reveals that pep4 is involved in dimerization of cell membrane-associated NKp46**

FRET occurring between FRET-compatible fluorophores is critically dependent upon the distance between them. Therefore, measurement of FRET efficiency between fluorescently labeled molecules has become a valuable tool for monitoring intermolecular distances and interactions in cells (44–46). To harness the FRET technique for evaluation of NKp46 dimerization, HEK293T cells were cotransfected with fluorescently labeled NKp46-CFP and NKp46-YFP constructs (Fig. 6A) and seeded on either uncoated coverslips or coverslips coated with an Ab to NKp46, mimicking a ligand to NKp46. The cells seeded on uncoated coverslips exhibited a measurable level of constitutive FRET efficiency of 23% (Fig. 6B, 6C). This is probably due to random proximity and interactions of the CFP- and YFP-labeled transfected NKp46 molecules in the cell membrane. However, seeding the cells on the coverslips coated with the NKp46 ligand resulted in a significant increase in the FRET efficiency detected between NKp46-CFP and NKp46-YFP molecules (32.4%, Fig. 6B, 6C). Seeding the cells on the ligand-coated coverslips in the presence of a mock peptide did not affect FRET efficiency (34.7%). In contrast, pep4 strongly abrogated the effect of the NKp46 ligand on the inter-NKp46 FRET (22.7%), reducing it to the baseline level. This inhibition was not due to inference with the ligand binding to cell surface-expressed NKp46 as evident from Fig. 6D. These data demonstrate that ligand binding results in a strong increase in intermolecular interactions between NKp46 molecules in the cell membrane, indicating a ligand-induced NKp46 dimerization, while pep4 negates this effect.

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

**FIGURE 4.** A model of the D2 dimer. Two D2 monomers are shown in different colors (red and blue). In each monomer, the sequence corresponding to pep4 is colored in yellow. Although the contact surfaces contributed from different D2 monomers are largely symmetrical, only one pair of potential interactions is shown for clarity. Residues contributed from different monomers are labeled with or without asterisks. A cluster of hydrophobic residues at the interface in the C-term part of the monomers is shown in Van der Waals representation and colored in a residue-specific mode. Interdomain salt bridges observed in the course of the molecular dynamics simulation are indicated.

### Table I. Pep4 mutagenesis reveals the necessity of Arg145–Asp147 for pep4 inhibition ability

| 136 | 137 | 138 | 139 | 140 | 141 | 142 | 143 | 144 | 145 | 146 | 147 | 148 | 149 | 150 | 151 | 152 | 153 | 154 | 155 | Percent of Inhibition ± SD, % |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| pep4 | S | G | E | K | V | T | F | Y | C | R | L | D | T | A | T | S | M | L | L | 81 ± 3.9 |
| Mut1 | A | | | | | | | | | | | | | | | | | | | | 0.1 ± 0.1 |
| Mut2 | | | | | | | | | | | | | | | | | | | | | 1.4 ± 0.4 |
| Mut3 | A | A | | | | | | | | | | | | | | | | | | | 87 ± 0.1 |
| Mut4 | A | A | A | | | | | | | | | | | | | | | | | | 81 ± 2.9 |
| Mut5 | | | A | A | | | | | | | | | | | | | | | | | 59 ± 28 |
| Mut6 | A | A | | | | | | | | | | | | | | | | | | | 79 ± 0.2 |
| 15mer | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 86 ± 1.6 |
| 13mer | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | — | 0.6 ± 0.8 |

Inhibition of rNKp46D2 binding to HeLa cells in the presence of pep4 and pep4-mutants. Results are shown as percent of inhibition ± SD that summarize two to five repetitive experiments for each pep4 mutant. Percent of inhibition was calculated compared with reference positive staining by the NK receptor-Ig without peptides (see Materials and Methods). 15mer represents the 15mer-shared core sequence of pep4 and pep5. Results showing loss of inhibition are set in boldface. A, Substitution with alanine; –, amino acid omission.
Discussion

The membrane-proximal extracellular domain of NKp46 (NKp46D2) was identified as the ligand-binding domain of the NKp46 receptor (28). In the current study, we dissected NKp46D2 into overlapping peptides to identify the ligand binding sites. We were unsuccessful in defining these sites; nevertheless, we revealed the epitope that is involved in NKp46D2 dimerization and demonstrate its involvement in NKp46-mediated lysis.

We first identified that an NKp46D2-derived peptide (aa 136–155, designated as pep4) specifically inhibited the binding of rNKp46 to target cancer cells (Fig. 1). R145 and D147 were essential for pep4 function, and a 15mer core sequence sufficed to inhibit binding (Fig. 1, Table I). We then tested whether the pep4 sequence might be involved in the formation of the ligand binding site of NKp46. HS was reported as a ligand for NKp46 (16, 27). Yet, pep4 inhibition of rNKp46 binding to target cells was not affected by the presence of membrane-associated HS or by the rNKp46 ability to bind HS (Fig. 3A, 3B). Furthermore, pep4 did not bind to target cancer cells (Fig. 3C) but showed specific interaction with rNKp46D2 itself (Fig. 3D). We thus suspected that pep4 could affect rNKp46 binding to target cells through an allosteric effect and hypothesized that pep4 contains amino acids involved in NKp46 dimerization. Allosteric modulators have thus far been considered as molecules binding to an allosteric site distinct from that of the reference ligand (orthosteric site). The concept of allosteric peptide modulators affecting receptor binding through inhibition of dimerization is well characterized for G protein-coupled receptors (47). In this framework, pep4’s inhibition of rNKp46 binding to target cells could be explained by its interference with the formation of dimers between two NKp46D2 domains in one recombinant NKp46-Ig molecule. In addition, pep4 could interfere with dimers produced between adjacent rNKp46 molecules in solution. The interference with rNKp46 dimerization by pep4 would lead to reduced avidity followed by reduced binding. On the recombinant NKp46 level, we showed that the R145Q mutation significantly reduces both rNKp46 binding to tumor cells and the effect of pep4 on rNKp46 binding (Fig. 5). Physiologically, we showed that pep4 inhibits lysis and degranulation by a primary human NK cell and that Abs to NKp46 annulled this inhibition (Fig. 2). Therefore, if pep4 interferes with NKp46 dimerization, then NKp46 cell surface dimerization contributes to NKp46-mediated lysis of target cells. The notion that homodimerization is imperative to the function of cell surface receptors was proved for various receptors, including NK receptors such as NKG2D (48, 49).

Notably, pep4 did not bind to NKp46-expressing NK cells unless HS-binding peptide was added (Fig. 3E). We hypothesize that NK-expressed HS could mask the NKp46-pep4 region in NK-expressed NKp46 and that addition of excess HS-binding peptide displaces the NKp46-bound HS and exposes the NKp46-pep4 region to interact with the exogenously added pep4. Indeed, the pep4 region and the HS binding site are both in NKp46D2, and the pep4 region is proximal to the HS binding site (27). This hypothesis then leads to the question of NKp46 unmasking during the formation of the immunological synapse. It is intriguing to consider the 2B4–CD48 interaction, essential for NKp46-mediated function, as the mechanism involved in HS unmasking through the HS-binding function of CD48.

To test pep4’s inhibition of NKp46 dimerization in a cellular context, we used a FRET-based approach. FRET measurements have been successfully used previously to track interactions between membrane proteins in immunological synapses of T cells, B cells, and NK cells (50–53). The increase in FRET efficiency between fluorescently labeled receptor molecules has been usu-

FIGURE 5. Effect of pep4 on staining with rNKp46-R145Q. (A and B) HeLa cell lines were stained with 2 μg/well of rNKp46 and rNKp46-R145Q. (C) HeLa cell lines were coincubated with 2 μg/well of rNKp46D2, rNKp46, and rNKp46-R145Q in the presence of 10 μg/well pep4, 15mer-shared sequence, or mock peptides. Reduced staining of NK receptor-Igs mediated by the presence of peptides is shown as percent inhibition compared with staining without peptides. The results are a summary of three experiments (B) and from one representative experiment of three performed (A, C). Bars, ±SD. *p ≤ 0.01.
ally attributed to the crowding effect produced by multivalent ligands. A bivalent NKp46 ligand (anti-NKp46) used in the FRET assay (Fig. 6) could also produce a crowding effect, which would account for the increase in FRET efficiency upon NKp46 ligation. However, pep4 inhibits the ligand-induced FRET increase without interfering with the ligand’s ability to bind NKp46 (Fig. 6D). Therefore, the observed increase in FRET is more likely due to NKp46 dimerization, which is blocked by pep4.

Pep4 is derived from NKp46D2. The D2 domain may therefore take part in the formation of the dimer’s interface. Consequently, the question of monomer orientation within the NKp46 dimers is of importance. Among the members of the NCR family, crystal structures are available for the ectodomains of NKp30 and NKp44 receptors (21, 24). Unlike the two C2-type Ig domains forming the NKp46 extracellular portion (25, 26), the ectodomains of NKp30 and NKp44 consist of a single I- or V-type Ig domain, respectively. According to their crystal structures, two ectodomains interact to form an antiparallel homodimer (21, 24). A multiple sequence alignment (ClustalW2) revealed that NKp46 shares only 7 or 4% identity to NKp30 or NKp44, respectively (data not shown). The low degree of sequence identity limits the use of NKp30 and NKp44 structural data in predicting the quaternary structure of NKp46.

Recently, a crystal structure of the ectodomain of member 1 of leukocyte Ig-like receptor subfamily B (LIR1) has become available (PDB code 1VDG). Akin to NKp46, the ectodomain of this receptor consists of two Ig-like domains, and it shares 38% sequence identity to the ectodomain of NKp46, rendering it a potential template for NKp46 homology modeling. According to the crystal structure, the two L-shaped LIR1 ectodomains form a “head to tail” dimer, in which the membrane-proximal domain (analogous to NKp46D2) of one monomer interacts with the distal domain of another monomer. Homology modeling of the NKp46 ectodomain using LIR1 as template produces a structure in which NKp46D2 sequences corresponding to pep4 are separated from each other by more than 50 Å and make no contribution to the formation of the dimer interface (data not shown).

Therefore, to acquire better insights into the structural organization of the NKp46D2 dimers, we turned to molecular docking. A rigid docking of the D2 domains extracted from the crystal structure of monomeric NKp46 ectodomains (PDB code 1oll) followed by a flexible docking and a short molecular dynamics simulation produced a low-energy docking solution indicating the formation of parallel D2 homodimers (C2 symmetry). In the structure, the sequences corresponding to pep4 are situated at the dimer’s interface and form direct interactions with each other. In particular, R145 forms a salt bridge with D147 from the opposite

FIGURE 6. FRET analysis of pep4 effect on dimerization of cell surface-associated NKp46. Pep4 inhibits a ligand-induced increase in FRET efficiency between fluorescently labeled NKp46 molecules. (A) Schematic representation of the proposed ligand-induced NKp46 dimerization and its effect on FRET efficiency between fluorescently labeled NKp46 molecules. (B) Representative confocal images of cells coexpressing NKp46-CFP and NKp46-YFP that were seeded on anti-NKp46 mAb-coated or uncoated coverslips in the presence of the peptides as indicated. FRET efficiency values are presented in a pseudo-colored scale, with black color representing saturated pixels. Scale bars, 5 μm. (C) Graphical summary of FRET efficiency values with each bar representing an average ± SE. *p < 0.05, **p < 0.01. (D) Pep4 does not inhibit binding of anti-NKp46 to its target. HEK293T cells, cotransfected with NKp46-CFP and NKp46-YFP, were stained with anti-NKp46 mAb in the presence of the indicated peptides; intensity was determined by flow cytometry using fluorescent allophycocyanin-conjugated F(ab’)2 goat anti-mouse IgG.
monomer. Taking our experimental results and the results of the docking study into account, we propose a mechanism for the formation of NKp46 dimers, in which the proximal D2 domains associate in a parallel fashion bringing the intracellular (and transmembrane) NKp46 domains into close proximity.

The difference between the “head to tail” D2–D1 dimerization suggested by homology modeling and our experimental results supported by the D2-docking studies could represent alternative forms of NKp46 dimerization that are associated with resting and activation states of the receptor. In the crystal structure of the soluble monomeric NKp46 ectodomain (25, 26), the region corresponding to pep4 is in the direction opposite to D1 and could participate in a homodimerization process with another NKp46D2 ectodomain, resulting in adjacent intracellular domains that could facilitate functional association with intracellular effector molecules and enhance downstream signal transduction. This idea is consistent with our experimental data showing that pep4 interferes with NKp46-mediated lysis by NK cells.

To summarize, we identified the NKp46-dimerization epitope and showed that it is essential for lysis induced by NK. Modulation of NKp46 homodimerization and function by pep4 could be further explored for therapeutic intervention in cases in which NKp46-mediated lysis contributes to the pathology (54).

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: **Incubation with pep4 derived from NKp46 inhibits target cells lysis by NK cells.**

The cytotoxic activity of NK cells against various targets was assayed in 5hr $^{35}$S-Methionine release assays. $^{35}$S-labeled 721.221 target cells were co-incubated with human primary NK cells (A) and $^{35}$S-labeled CHO-K1 cells were co-incubated with NKp46-expressing NK-92 cell line (B) at 40:1 E:T ratio in the presence of pep4 or mock peptide. NKp46 expression for NKp46-expressing NK-92 and human primary NK cells is shown in the corresponding insets. The lysis levels were calculated by the following equation: % of specific lysis = [(cpm experimental well - cpm spontaneous release)/(cpm maximal release - cpm spontaneous release)] X 100. The results are from one representative experiment out of 2 experiments performed. Bars, ±SD. * P-value < 0.05, ** P-value < 0.01.