

NKp44 Triggers NK Cell Activation through DAP12 Association That Is Not Influenced by a Putative Cytoplasmic Inhibitory Sequence¹

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NKp44 (NCR2) is a member of the natural cytotoxicity receptor (NCR) family that is expressed on activated human NK cells. We dissected structural attributes of NKp44 to determine their contributions to receptor function. Our results demonstrate that surface expression and NK cell activation by NKp44 is mediated through noncovalent association with the immunoreceptor tyrosine-based activation motif-containing protein, DAP12. Physical linkage to DAP12 requires lysine-183 in the NKp44 transmembrane domain. Intriguingly, the cytoplasmic domain of NKp44 also contains a sequence that matches the immunoreceptor tyrosine-based inhibitory motif (ITIM) consensus. By expressing a chimeric receptor in an NK-like cell line, we found that this ITIM-like motif from NKp44 lacks inhibitory capacity in a redirected cytotoxicity assay. The NKp44 cytoplasmic tyrosine was efficiently phosphorylated in the chimeric receptor upon treating the cells with pervanadate, but it was unable to recruit ITIM-binding negative effector phosphatases. We also generated NK-like cell lines expressing epitope-tagged wild-type or tyrosine to phenylalanine mutant (Y238F) versions of NKp44 and compared their capacities to induce activation marker expression, promote IFN- γ production, or stimulate target cell cytotoxicity. We did not detect any tyrosine-dependent reduction or enhancement of NK cell activation through wild-type vs. Y238F mutant NKp44. Finally, the cytoplasmic tyrosine-based sequence did not provide a docking site for the AP-2 clathrin adaptor, nor did it potentiate receptor internalization. In summary, all activating properties and surface expression of NKp44 are mediated through its association with DAP12, and the putative ITIM in the NKp44 cytoplasmic domain does not appear to attenuate activating function. *The Journal of Immunology*, 2004, 172: 899–906.

The NKp44 receptor (NCR2) is a member of the natural cytotoxicity receptor (NCR)⁴ family of activating NK cell receptors that are important for tumor target cell recognition by NK cells during natural cytotoxicity responses (1–3). To date, the *NKp44* gene has only been identified in humans, while genes for the other NCR members, *NKp30* and *NKp46*, have also been described in rodents (4–6). Nonetheless, Southern blotting with the human *NKp44* gene revealed a cross-hybridizing band in mice (2), suggesting that rodents may indeed express an equivalent receptor. Interestingly, expression of NKp44 is restricted to only activated NK cells (1, 2), while the other NCR members are constitutively expressed by either resting or activated NK cells. This expression pattern suggests that NKp44 may contribute to the en-

hanced cytolytic capacity of IL-2-activated NK cells. It should also be noted that some TCR- $\gamma\delta$ clones were found to express NKp44 (2), suggesting that a subset of T cells may also express this receptor. Anti-NKp44 mAb can reduce NK cell cytotoxicity toward certain tumor target cells, thereby indicating that these targets express an appropriate ligand for the receptor (1, 6). The identity of this ligand on tumors is currently unknown, although viral hemagglutinins have been reported to interact with sialic acid determinants on NKp44 and another NCR, NKp46, to promote cytolysis of influenza virus-infected target cells (7, 8). NKp44 is a type I transmembrane protein that contains a single V-type Ig-superfamily extracellular domain and is noncovalently associated in the plasma membrane with a disulfide-linked homodimer of the DNAX-activation protein of 12 kDa (DAP12; also known as killer cell-activating receptor-associated protein (KARAP)) (1, 2). DAP12 is a transmembrane accessory protein that contains an immunoreceptor tyrosine-based activation motif, which transduces intracellular activation signals. Despite its interesting characteristics, particularly its unique expression on only activated human NK cells, little is known regarding the signal transduction properties of NKp44, other than its apparent association with DAP12.

Interestingly, the predicted protein sequence of the NKp44 intracytoplasmic domain (GenBank accession number AJ225109) contains a tyrosine-based sequence (EILY₂₃₈HTVA), which matches the context of an immunoreceptor tyrosine-based inhibitory motif (ITIM; V/IxYxxL/V). The presence of an ITIM is uncharacteristic for an activating receptor, because ITIM sequences are critical negative signaling elements on many inhibitory receptors. Phosphorylation of an ITIM tyrosine serves as a docking site for Src homology 2 (SH2) domains of SH2 domain-containing protein tyrosine phosphatase (SHP)-1, SHP-2, or SH2 domain-containing 5'-inositol phosphatase (SHIP), which mediate inhibitory function. We hypothesized that the ITIM-like sequence of

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⁴ Abbreviations used in this paper: NCR, natural cytotoxicity receptor; KIR, killer cell Ig-like receptor; wt, wild type; SH2, Src homology 2; SHP, SH2 domain-containing protein tyrosine phosphatase; SHIP, SH2-containing 5'-inositol phosphatase; ITIM, immunoreceptor tyrosine-based inhibitory motif; PVDF, polyvinylidene difluoride; IRES, internal ribosome entry site; EGFP, enhanced green fluorescent protein.

NKp44 could contribute inhibitory capacity that affects activation signaling through the receptor. We were particularly intrigued with this hypothesis upon finding the reports of two alternative NKp44 cDNAs (NKp44RG1 and NKp44RG2) in the GenBank/EMBL databases (accession numbers AJ010099, AJ010100, and AL136967.5; unpublished observations), which contain a frame shift that alters this cytoplasmic ITIM-like sequence, suggesting the existence of functionally divergent receptor isoforms. Importantly, the cytoplasmic tyrosine is situated near upstream acidic amino acids (glutamic acids at positions -3 and -5) and a hydrophobic residue at position +3 (valine), indicating that it can likely serve as an appropriate substrate for protein tyrosine kinases, particularly those of the Src family (9, 10).

In this report, we examined whether specific sequence elements on NKp44 contribute to its activating function. Our results demonstrated that NKp44 surface expression requires physical association with DAP12, which is dependent upon the integrity of a transmembrane lysine on NKp44. We also tested whether the putative ITIM sequence in NKp44 exhibits inhibitory capacity and/or influences the activation signals derived through the receptor. Our experiments indicate that, in isolation, the tyrosine-based sequence from the NKp44 cytoplasmic domain does not possess the capacity to contribute inhibitory function. Accordingly, the tyrosine-phosphorylated cytoplasmic domain did not recruit SHP-1, SHP-2 or SHIP phosphatases. In addition, phenylalanine mutation of the ITIM-like tyrosine in full-length NKp44 did not influence numerous activating functions of the receptor. We also found that the cytoplasmic tyrosine motif does not influence NKp44 internalization nor bind the AP-2 clathrin adaptor. In conclusion, our data indicate that all known activation through NKp44 can be attributed to the noncovalent association with the DAP12 homodimer.

Materials and Methods

Cell lines and Abs

NK-92 cells were grown in α -MEM supplemented with IL-2 as previously described (11). Phoenix-Amphotropic retroviral packaging cells were kindly provided by Garry Nolan (Stanford University, Stanford, CA) and grown as described (11). Jurkat cells were grown in RPMI 1640 medium supplemented with 10% FBS. DX9 (anti-KIR3DL1), 3.4.3.13 (anti-NKp44), and B159 (anti-CD56) mAbs were purified with protein G from hybridomas obtained from Drs. L. Lanier (University of California, San

Francisco, CA), M. Colonna (Washington University, St. Louis), and B. Perussia (Thomas Jefferson University, Philadelphia, PA), respectively. PE-conjugated DX9 was purchased from BD Pharmingen (San Diego, CA). The anti-FLAG mAb, M2, was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-DAP12 Ab was produced toward a recombinant form of the human DAP12 cytoplasmic domain and purified on protein A by Exalpha Biologicals (Boston, MA). PE-conjugated goat anti-mouse κ light chain Ab was obtained from Southern Biotechnology Associates (Birmingham, AL).

Cloning of cDNA constructs

cDNA constructs were prepared by PCR from a NK cell cDNA library using the synthetic oligonucleotides shown in Table I to generate wild-type, chimerized, or mutated NKp44 and KIR3DL1 receptor cDNAs with engineered endonuclease restriction sites, as marked in Fig. 2A. Full-length or truncated NKp44 constructs were engineered with the NKp44 leader, the FLAG-epitope tag sequence, and the NKp44 protein sequence. Transition amino acid sequences between leader, FLAG, and amino terminus of full-length NKp44 is F₆PGSQADYKDDDDKLQSKAQV₆ (FLAG sequence underlined, outside residues numbered (2), and italic leucine encoded by *Hind*III restriction site). The cDNA for 3DL1.p44 chimeric receptors consisted of the KIR3DL1 sequence until methionine-373 in the cytoplasmic domain, a *Bsp*HI site (as described in Ref. 11), and the cytoplasmic domain of NKp44 from leucine-226 (Fig. 2A). Transition between KIR3DL1 and NKp44 is **K₃₆₇KNAAVM**LRSL₂₀₈ (KIR3DL1 in bold, NKp44 underlined, asparagine encoded by *Bsp*HI site in italics, and representative amino acids in each protein numbered). Tyrosine-238 in the cytoplasmic domain of NKp44 was mutated to phenylalanine (Y238F constructs; numbering as in Ref. 2). The cDNA constructs were ligated into the pBMN-IRES-EGFP plasmid (from G. Nolan) or a modified version lacking the internal ribosome entry site (IRES) and enhanced green fluorescent protein (EGFP) sequences (pBMN-NoEGFP) as previously described (11). The KIR3DL1 construct was previously described (11). The cDNAs of DAP12/KARAP, DNAX-activation protein of 10 kDa (DAP10; also known known as PI-3 kinase-associated protein of 10 kDa (KAP10)), and Fc ϵ RI γ accessory proteins were cloned into pBMN-IRES-EGFP using the oligos listed in Table I. DAP12/KARAP and Fc ϵ RI γ were cloned from a cDNA library, and DAP10/KAP10 was cloned from a His-tagged cDNA construct in the pJFE14 vector that was kindly provided by John Trowsdale and Julie Fairbanks (Cambridge University, Cambridge, U.K.). All PCR were performed using Platinum Pfx DNA polymerase (Life Technologies, Rockville, MD), and the integrity of all constructs was confirmed by automated sequencing in the Fox Chase Cancer Center (FCCC) DNA Sequencing Facility.

Retroviral transduction

Retrovirus was prepared as previously described (11). Briefly, the Phoenix packaging line was transfected with pBMN vectors, and culture supernatant was harvested 2 days later. NK-92 cells were incubated with viral

Table I. Sequences of synthesized oligonucleotide primers to make constructs in this study^a

Primers	Sequences (5'-3')
1) NKp44 sense from ATG start (<i>Eco</i> RI)	AGCTAGAATTCAACATGGCCTGGCGAGCCCT
2) NKp44 antisense leader + FLAG tag (<i>Hind</i> III)	TAGCAAGCTTGTGTCATCGTCTTGTAGTCTGCCTGAGAGCCTGGGA
3) Wild type NKp44 sense from after leader (<i>Hind</i> III)	ATCGTAAAGCTTCAATCCAAGGCTCAGGTA
4) Wild type NKp44 antisense to stop (<i>Not</i> I)	AAGCTAGCGCCGCTCACAAGTGTGTTTCATCATC
5) Mutant NKp44 ITIM sense	GAGAGAGAAATATTTTACACTGTTGCA
6) Mutant NKp44 ITIM antisense	TGCAACAGTGTGAAATAATATTTCTCTCTC
7) Truncated NKp44 antisense + stop (<i>Not</i> I)	TGCATGCGGCCGCTACTCCATCAGCGTTT
8) KIR3DL1 sense from ATG start (<i>Bam</i> HI)	TCGACTGGATCCACCATGTCGCTCATGGTCTGTCAGCATG
9) KIR3DL1 antisense to cytoplasmic (<i>Bsp</i> HI)	AGGCTCTTGGTTCATGACAGCAGCAT
10) NKp44 sense cytoplasmic 3DL1 fusion (<i>Bsp</i> HI)	ATGCTGCTGTCATGACCTCAGGAGCCTGGATAC
11) NKp44 sense cytoplasmic GST fusion (<i>Eco</i> RI)	GATATCGAATTCCTGGGGGGACATATGGTGGA
12) Sense NKp44 K183A mutation	ACTCCTCGTAGCCCGAGCCTGGTGTCT
13) Antisense NKp44 K183A mutation	AGCACAGGCTCGCGGCTACGAGGAGT
14) Sense NKp44 cytoplasmic Gal4 fusion (<i>Eco</i> RI)	GATATCGAATTCCTGGGGGGACATATGGTGGA
15) Sense Fc ϵ RI γ from ATG start (<i>Bam</i> HI)	ATCAGTGGATCCAAGATGATTCAGCAGT
16) Antisense Fc ϵ RI γ to stop (<i>Not</i> I)	ACTGTAGCGGCCGCTACTGTGGTGGTTTCTCA
17) Sense DAP12/KARAP from ATG start (<i>Bam</i> HI)	ATCAGTGGATCCGTCATGGGGGGACTTGAACT
18) Antisense DAP12/KARAP to stop (<i>Xho</i> I)	TCATACTCGAGTCAATTTGTAATACGGCCTCTGT
19) Sense pJFE14 vector for DAP10/KAP10 (<i>Xho</i> I)	ATCGTGAGGATCCGCTTTACTTCTAGGCCTGTAC
20) Antisense pJFE14 vector for DAP10/KAP10 (<i>Not</i> I)	ATTGTACGCGGCCGCTCGACGGAAATTCAGC

^a Oligo numbering corresponds with schematic location as in Fig. 2A. Names of engineered restriction endonuclease cleavage sites are listed in parentheses and corresponding sequences are underlined.

supernatant for 8 h with Lipofectamine Plus (Life Technologies), and cells expressing EGFP and/or surface receptor were sorted 3 days later on a FACS Vantage^{SE} (BD Biosciences, Mountain View, CA) in the FCCC Cell Sorting Facility. Surface expression of transduced receptors was stable for at least 2 months. To cotransduce NKp44 and accessory proteins into Jurkat cells, the Jurkat parent was transduced with retrovirus containing cDNA for either DAP12/KARAP, DAP10/KAP10, or FcεRIγ in pBMN-IRES-EGFP. Transduced cells were sorted for EGFP 5 days later and subsequently transduced with retrovirus containing the FLAG-NKp44 cDNA in the pBMN-NoEGFP vector. The entire cell population was analyzed 4 days later by staining for surface expression with anti-FLAG mAb.

Redirected cytotoxicity assay

Receptors transduced into NK-92 cells were tested for impacts on killing in a redirected cytotoxicity assay using the FcγR⁺ P815 murine mastocytoma target cell line as previously described (11). NK-92 cells were either resting (4 days after IL-2 addition) or stimulated (1 day after IL-2) to test activating or inhibitory properties of the full-length or chimeric receptors, respectively. Cytotoxicity was measured in triplicate determinations using a standard 4-h ⁵¹Cr release assay with or without Abs (5 μg/ml) to engage the KIR3DL1 extracellular domain (DX9), FLAG-tagged NKp44 (M2), or the control receptor CD56 (B159).

Cell surface activation marker expression assay

Six-well plates were coated with mAbs (10 μg/ml HBSS overnight at 4°C or 2 h at 37°C) to engage receptors, washed, and seeded with 2 × 10⁶ resting NK-92 cells (day 4 after IL-2) in 5 ml of complete medium. Cells were stimulated for 18 h before harvest by harsh pipetting, washed on ice, and labeled with PE-conjugated Abs to CD69, CD25, or CD95L (BD PharMingen) in the presence of azide. Cells were analyzed on a FACScan analyzer (BD Biosciences).

IFN-γ assay

Receptor-transduced NK-92 cells (2 × 10⁶) were stimulated with Abs coated on six-well plates (5 ml/well) as described above for 18 h. Supernatants were harvested and assayed for IFN-γ production using an ELISA kit (BD PharMingen).

Metabolic labeling, immunoprecipitation, and immunoblotting

For metabolic labeling, NK-92 cells (160 million) were washed twice in HBSS and cultured for 75 min in 5 ml of cysteine/methionine-free DMEM (Sigma-Aldrich) containing 5 mCi [³⁵S]cysteine/methionine (Promix; Amersham Pharmacia Biotech, Piscataway, NJ). Labeled cells were washed once with HBSS. Some cell samples were treated for 10 min with pervanadate (100 μM sodium orthovanadate plus 10 mM H₂O₂). Cells were lysed for 30 min on ice in 1 ml of buffer containing 1% of either Triton X-100, digitonin, Brij 58 (polyoxyethylene 20 cetyl ether) (all from Sigma-Aldrich), sodium *n*-dodecyl-β-D-maltoside, or *n*-octyl-β-D-glucopyranoside (octyl glucoside) (both from Calbiochem, La Jolla, CA) as previously described (11). Detergent-insoluble debris was removed from lysates by centrifugation at 20,800 × *g* for 15 min at 4°C. Lysates were then pre-cleared twice for 30 min each at 4°C with protein G-coupled agarose (Upstate Biotechnology, Lake Placid, NY). FLAG-tagged NKp44 was immunoprecipitated for 90 min at 4°C with 30 μl of M2 mAb-Sepharose beads (1 mg mAb/ml cyanogen bromide-conjugated Sepharose 4 Fast Flow; Amersham Pharmacia Biotech), separated on SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA), and probed with rabbit anti-DAP12 Ab (0.25 μg/ml), rabbit anti-SHP-1 Ab (1 μg/ml; Upstate Biotechnology), rabbit anti-SHP-2 Ab (1 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-SHIP (1 μg/ml; Santa Cruz Biotechnology) plus HRP-conjugated protein G (Calbiochem), anti-FLAG mAb (M2) plus HRP-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), or HRP-conjugated anti-phosphotyrosine mAb (4G10; Upstate Biotechnology). Immunoblotted proteins were visualized by chemiluminescence using the ECL detection reagents (Amersham, Arlington Heights, IL).

Yeast two-hybrid system

The yeast two-hybrid vectors pPC62 and pPC86 were obtained from P. Chevray and D. Nathans (Johns Hopkins University, Baltimore, MD) (12), and use of the yeast two-hybrid system was described previously (13, 14). Receptor cytoplasmic domain bait constructs for human KIR2DL1 (aa 265–348), human ICAM3 (CD50; aa 482–518), and human NKp44 (aa 220–276) were generated by PCR and ligated into a modified version of the pPC86 plasmid (named pPC87) in which the Gal4 DNA binding domain fusion cassette from pPC62 was introduced to replace the transcrip-

tional activation cassette. In this way, pPC87 produced a Gal4 DNA binding fusion protein in a plasmid under *TRP1* selection, which is compatible with the pACT vector encoding the Gal4 transcriptional activation domain fused with aa 3–436 of the μ2 (AP50) adaptor protein under *LEU2* selection (construct kindly provided by Chander Raman, University of Alabama, Birmingham, AL (15)). A construct encoding the Gal4 DNA binding domain fused to the cytoplasmic domain of murine CD5 (aa 378–471) in pGBT9 plasmid (*TRP1* selection; Clontech, Palo Alto, CA) was kindly provided by C. Raman (15). The reporter yeast strain was AH109 (BD Biosciences), which contained two integrated growth selection reporters (*His3* and *Ade2*). Both reporters contain upstream LexA operators that are each believed to bind two LexA hybrid proteins. Yeast culture conditions, transfection protocol, and chemiluminescence assay for β-galactosidase were performed as previously described (14).

Results

DAP12 association with NKp44 is required for receptor surface expression

The DAP12/KARAP accessory protein was previously reported to co-immunoprecipitate with NKp44 from digitonin lysates of NK cells (1, 2). We tested this association in NK-92 cells that had been metabolically labeled with [³⁵S]cysteine/methionine and lysed with digitonin (1, 2). The receptor complex was immunoprecipitated with a mouse anti-NKp44 mAb, 3.43.13 (kindly provided by Marco Colonna, Washington University, St. Louis). We confirmed that the mAb can immunoprecipitate NKp44 in association with DAP12, but our experiments showed only weak co-immunoprecipitation of DAP12 from digitonin lysates as assessed by immunoblotting (Fig. 1). The results prompted us to further examine the capacities of other mild nonionic detergents to optimally release NKp44 and retain the DAP12 association. When directly compared, dodecyl maltoside proved to be a significantly better detergent than digitonin for this purpose (Fig. 1). In contrast, octyl glucoside only weakly solubilized the receptor and Brij 58 did not detectably release NKp44 from the plasma membrane (Fig. 1). Therefore, we confirmed that DAP12 is associated with NKp44 in

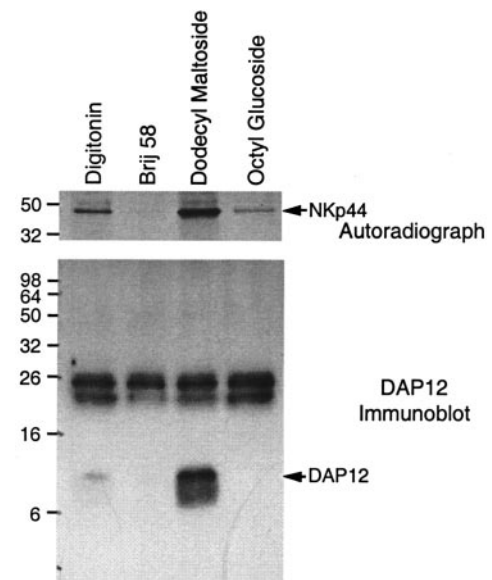


FIGURE 1. Dodecyl maltoside detergent optimally releases DAP12-associated NKp44. NK-92 cells (45 million/sample) were metabolically labeled with [³⁵S]cysteine/methionine, and aliquots were lysed in buffers containing 1% of the indicated detergents. NKp44 was immunoprecipitated from cleared lysates, separated on 15% SDS-PAGE under reducing conditions, transferred to PVDF membrane, autoradiographed (*top*), and immunoblotted with anti-DAP12 Abs (*bottom*). DAP12 was not visible in the autoradiograph, presumably due to slow protein turnover.

NK cells, and we conclude that dodecyl maltoside is a substantially better detergent for solubilizing the intact receptor complex from NK cells.

We next addressed the molecular basis of DAP12 association and tested whether DAP12 association was required for surface expression of NKp44. Recombinant retrovirus engineered to co-express a FLAG-epitope tagged form of wild-type NKp44 (NKp44.wt; Fig. 2A) and EGFP was used to transduce the DAP12-expressing NK-like cell line, NK-92, and the DAP12-deficient T cell line, Jurkat. EGFP-expressing transduced cells were sorted and stained with anti-FLAG mAb to detect surface expression of NKp44.wt. Although NKp44.wt was strongly expressed on transduced NK-92 cells, the DAP12-deficient Jurkat T cell line could not support surface expression of NKp44 (Fig. 3A). Next, we tested whether DAP12 could rescue surface expression of NKp44 in Jurkat cells by co-expressing NKp44 in combination with either DAP12, DAP10, or FcεRIγ accessory proteins. As shown in Table II, co-expression of DAP12 supported surface expression of NKp44, while DAP10 or FcεRIγ did not. Parental Jurkat cells endogenously express TCR-ζ (data not shown), which indicates that TCR-ζ is also incapable of promoting surface expression of NKp44. We further mutated the transmembrane lysine residue in NKp44 (K183) to alanine to test whether it is required for DAP12 association and receptor surface expression. As shown in Fig. 3B, the K183A mutant was only marginally expressed on the surface of NK-92 cells, despite co-expression of DAP12. Furthermore, the NKp44.K183A mutant did not associate with DAP12 in dodecyl maltoside lysates, while the endogenous NKp44 (subsequently immunoprecipitated from the same lysates with the anti-NKp44 mAb, 3.43.13) was strongly associated with the accessory protein, as shown in Fig. 3C. Therefore, DAP12 association is required for cell surface expression of the receptor, and association is dependent upon integrity of lysine-183 of NKp44.

The tyrosine-based sequence in NKp44 does not function as an ITIM

We next addressed whether the ITIM-like sequence in the NKp44 cytoplasmic domain influences activating function of the receptor complex. To test the inhibitory capacity of the cytoplasmic domain, we generated a chimeric receptor in which the cytoplasmic domain, including the ITIM-like sequence of NKp44, was directly fused to the extracellular and transmembrane domains of KIR3DL1 to create a chimeric receptor, which was designated 3DL1.p44.wt (schematized in Fig. 2A). As a negative control, a 3DL1.p44 chimera was also produced in which the ITIM-like tyrosine of NKp44 (tyrosine-238) was mutated to phenylalanine (designated 3DL1.p44.Y238F). We have previously validated this chimeric killer cell Ig-like receptor (KIR) strategy as a powerful technique to demonstrate the strong inhibitory properties of the cytoplasmic domain of KIR2DL4 (11). The 3DL1.p44 chimeric receptors were expressed at similar levels on the surface of transduced NK-92 cells, as assessed by anti-KIR mAb staining (Fig. 2B).

As in our previous studies (11, 16), the inhibitory capacities of the 3DL1.p44 chimeric receptors were tested on IL-2-stimulated NK-92 cells in "redirected" cytotoxicity assays toward the FcγR⁺ P815 target cell, using the DX9 mAb as a surrogate ligand to specifically engage the chimeric receptors. DX9-mediated engagement of the wild-type KIR3DL1 receptor in this assay induced potent inhibition of cytotoxicity (Fig. 4A). In contrast, engagement of either 3DL1.p44 chimeric receptor did not influence the cytolytic response when compared with control cultures treated with anti-CD56 mAb or lacking added Ab (Fig. 4A). Inhibition was not evident even when NK-92 cells were tested under resting conditions (4 days after low-dose IL-2; Fig. 4A). These results indicated

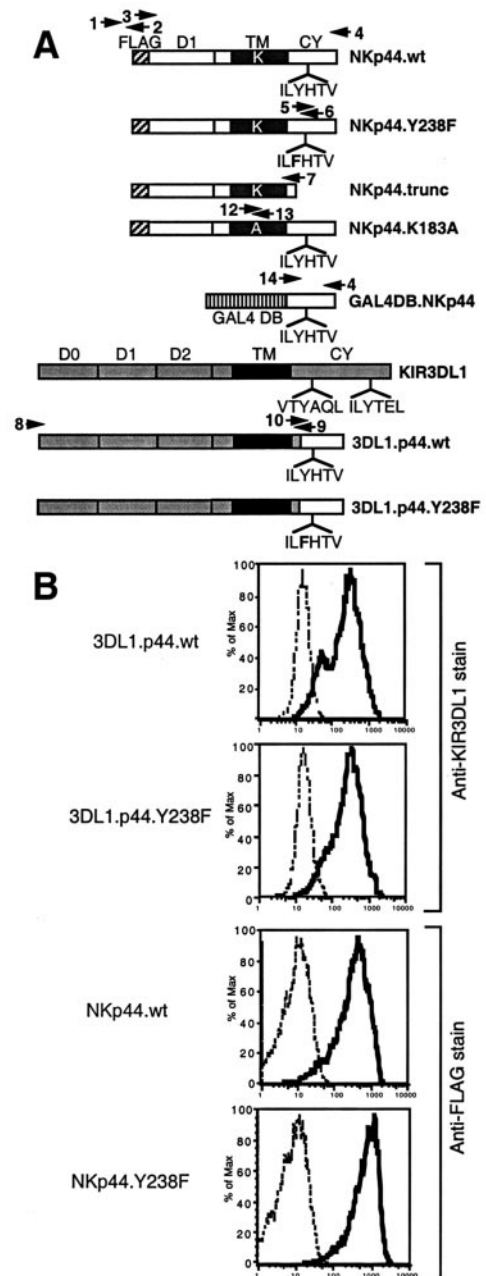


FIGURE 2. NKp44 constructs and their expression in the NK-92 cell line. *A*, Schematic diagram of protein products from cDNAs encoding FLAG-tagged and chimeric NKp44 receptors. Domains labeled include FLAG epitope tag, extracellular KIR Ig domains (D0, D1, D2), transmembrane domain (TM), and cytoplasmic domain (CY). The transmembrane lysine residue is marked (K or mutant A), as well as the tyrosine-based ITIM or ITIM-like sequences in the cytoplasmic domains. Numbered arrows represent locations of oligonucleotide primers (corresponding to those listed in Table I) used to synthesize the cDNAs. *B*, The NK-92 cell line was transduced with retroviral preparations encoding the FLAG-tagged or chimeric NKp44 receptors, and receptor-expressing cells were sorted by flow cytometry. Surface expression (solid histograms) was determined with either anti-FLAG mAb (M2 + PE-anti-mouse κ) or anti-KIR3DL1 mAb (PE-DX9) compared with unstained or secondary alone (dashed histograms).

that the tyrosine-based motif on NKp44 is not functioning as an ITIM.

We next performed biochemical analysis to test if the ITIM-like motif on 3DL1.p44.wt can be tyrosine-phosphorylated and if it can

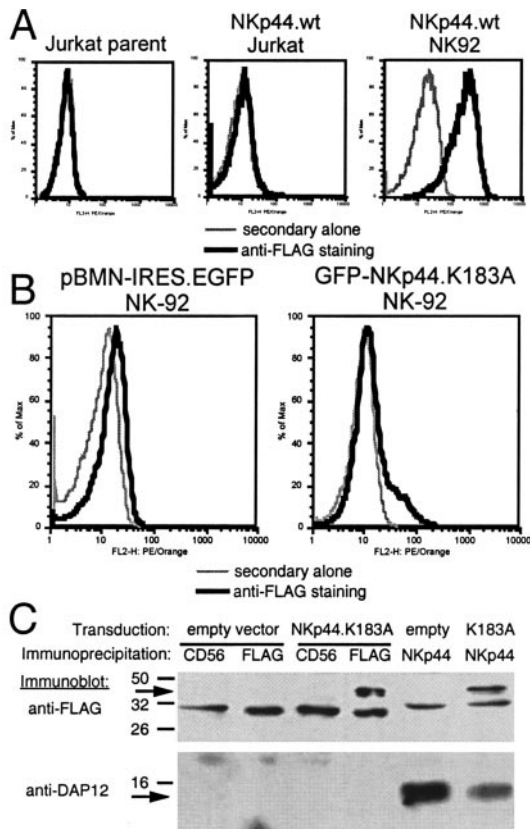


FIGURE 3. Surface expression of NKp44 requires association with DAP12 via the transmembrane lysine residue. *A*, NKp44 is not expressed on the surface of the DAP12-deficient T cell line, Jurkat. Jurkat T cells or NK-92 cells were transduced with FLAG-NKp44.wt, and EGFP-positive transduced cells were sorted and stained for surface expression with the anti-FLAG mAb (M2). *B*, Mutation of the transmembrane lysine on NKp44 to alanine significantly disrupts surface expression in NK-92 cells. NK-92 cells were transduced with a FLAG-tagged form of NKp44 in which the transmembrane lysine was mutated to alanine (K183A) or the empty vector (pBMN-IRES-EGFP), EGFP⁺ cells were sorted, and these were stained with anti-FLAG mAb. *C*, The K183A mutant NKp44 does not associate with DAP12 in NK-92 cells. Transduced NK-92 cells shown in *B* were lysed in dodecyl maltoside (1%) detergent, and lysates were sequentially immunoprecipitated with anti-CD56, anti-FLAG, and anti-NKp44 mAbs. Immunoprecipitates were separated on 15% SDS-PAGE, transferred to PVDF membrane, and sequentially immunoblotted with anti-FLAG and anti-DAP12 Abs. Note that some FLAG-tagged NKp44 was still isolated in the final immunoprecipitation with anti-NKp44 (lane 6), but the endogenous receptor in that immunoprecipitate was associated with DAP12.

recruit any of the inhibitory SH2-containing phosphatases. Per-vanadate stimulation of NK-92 cells resulted in strong tyrosine phosphorylation of KIR3DL1.p44 at the predicted molecular mass of ~62 kDa (Fig. 4*B*). In contrast, the Y238F mutant chimeric receptor was not tyrosine-phosphorylated (Fig. 4*B*). Together, these results demonstrate that the ITIM-like sequence of NKp44 can be tyrosine-phosphorylated in the context of the chimeric receptor. We could not, however, detect co-immunoprecipitation of SHP-1, SHP-2, or SHIP with tyrosine-phosphorylated 3DL1.p44.wt (Fig. 4*B*; SHIP data not shown). On the contrary, our analyses have routinely shown that inhibitory KIR, such as 3DL1, strongly bind SHP-1 and/or SHP-2 under these conditions (11, 16). Therefore, the cytoplasmic tyrosine from NKp44 can serve as a protein tyrosine kinase substrate, but this phosphorylated tyrosine cannot serve as a docking site for SH2-containing phosphatases that are known effectors of negative signaling.

Table II. Expression of FLAG-NKp44 on the cell surface of Jurkat cells when cotransduced with accessory proteins^a

NKp44 Transduced:	-		+	
	Secondary only	Anti-FLAG	Secondary only	Anti-FLAG
Jurkat parent	0.15	0.41	0.17	1.15
Jurkat + DAP10	0.31	0.61	0.41	0.81
Jurkat + DAP12	0.39	0.34	0.39	11.8
Jurkat + FcεRIγ	0.42	0.77	0.26	0.75

^a Jurkat cells were first infected with recombinant retrovirus containing cDNAs for DAP10/KAP10, DAP12/KARAP, or FcεRIγ accessory proteins in the pBMN-IRES-EGFP vector and subsequently sorted for the EGFP-expressing transduced cells. The accessory protein-transduced cells (or Jurkat parent cells) were then secondarily infected with retrovirus containing the FLAG-NKp44 cDNA in the pBMN-NoEGFP vector. Cells were surface stained 4 days later with secondary alone (PE-conjugated anti-κ: 1:100) or anti-FLAG (10 μg/ml + secondary), and the percentage of cells exhibiting higher than background PE labeling are shown. Results are representative of two independent experiments. Typically 5-15% of infected cells are transduced under these conditions.

The tyrosine-based sequence in NKp44 does not affect activation properties of the receptor

Next, we tested whether the tyrosine-based sequence on NKp44 influences the activating properties of the full-length receptor. To study its influence, we mutated the ITIM-like tyrosine (Y238) to phenylalanine on the FLAG-epitope tagged NKp44 (to generate NKp44.Y238F) as shown in Fig. 2*A*. NKp44.wt and NKp44.Y238F were expressed at similar levels in NK-92 cells by retroviral transduction (Fig. 2*B*) and compared for functional activation capacities.

We first compared the abilities of these FLAG-tagged forms of NKp44 to stimulate the up-regulation of several cell surface activation markers in resting NK-92 cells in response to engagement with plate-bound anti-FLAG mAb. The activation markers CD25 (IL-2 receptor α-chain), CD69, and CD95L (FasL) were all increased to similar extents by engagement of either NKp44.wt or NKp44.Y238F (Fig. 5*A*). In contrast, engagement of the 3DL1.p44.wt chimera did not increase activation marker expression (Fig. 5*A*), which demonstrates that the tyrosine-based motif does not contribute direct activating capacity to receptor function. To confirm that the tyrosine-based sequence does not influence activation potential under weak stimulating conditions, we performed a dose-response comparison. As shown in Fig. 5*B*, similar CD69 up-regulatory capacities were also observed when both full-length NKp44 receptors were engaged with low doses of anti-FLAG mAb. Thus, the tyrosine-based sequence in NKp44 did not exhibit intrinsic activating properties, nor did it inhibit or potentiate the capacity of the receptor to trigger up-regulation of activation markers on NK-92 cells.

We next tested whether the presence of the tyrosine-based sequence could influence cytolytic or cytokine release responses through NKp44. NKp44.wt and NKp44.Y238F stimulated nearly identical levels of killing response by resting NK-92 cells when engaged with anti-FLAG mAb in a redirected cytotoxicity assay (Fig. 6*A*). Similarly, plate-bound anti-FLAG mAb stimulated similar levels of IFN-γ release when NK-92 cells expressed either of the receptors (Fig. 6*B*). In conclusion, our results indicate that the tyrosine-based sequence in the cytoplasmic domain of NKp44 does not influence activating function of the full-length receptor in assays of activation marker induction, cytotoxicity, or IFN-γ release.

The cytoplasmic domain of NKp44 does not associate with the AP-2 clathrin adaptor complex

Because signaling function of the ITIM-like sequence was not evident, we reasoned that the sequence could alternatively contribute

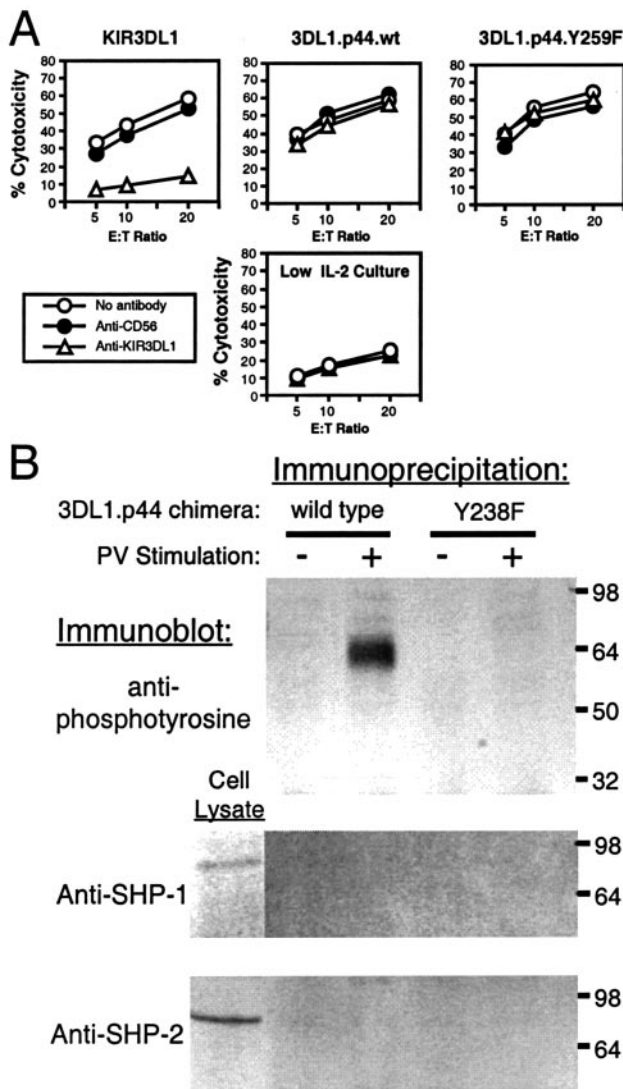


FIGURE 4. The cytoplasmic tyrosine-based sequence of NKp44 does not affect the killing of P815 target cells or bind SH2-containing protein tyrosine phosphatases. **A**, The cytoplasmic domain of NKp44 does not inhibit redirected killing. KIR3DL1 or KIR3DL1.p44 chimeric receptors (wild-type or Y238F mutant) were expressed in NK-92 cells and engaged with anti-KIR3DL1 Ab (DX9; Δ) during cytotoxicity of the Fc γ R⁺ P815 target cell at various effector-to-target (E:T) ratios. Control assays were performed in the absence of mAb (\circ) or with anti-CD56 mAb (B159; \bullet). NK-92 cells were stimulated with IL-2 on the day before assay to enhance their cytolytic capacity toward P815. The *lower panel* is an assay of KIR3DL1.p44.wt-transduced cells stimulated 4 days after low-dose IL-2 stimulation (half of normal amount). Percent of maximal cytotoxicity is indicated. **B**, The cytoplasmic tyrosine of NKp44 can be tyrosine-phosphorylated, but does not recruit SHP-1 or SHP-2 phosphatases. NK-92 cells transduced with KIR3DL1.p44.wt or KIR3DL1.p44.Y238F were either untreated (–) or pervanadate-treated (+) for 10 min before lysis in 1% Triton X-100 buffer and immunoprecipitation with anti-KIR3DL1 mAb (DX9). Immunoprecipitates were separated on 10% SDS-PAGE, transferred to PVDF membranes, and probed with anti-phosphotyrosine (4G10-HRP), anti-SHP-1, anti-SHP-2, or anti-SHIP Abs plus protein G-HRP secondary. SHIP was never observed to bind phosphorylated KIR3DL1.p44.wt (data not shown).

to the regulation of NKp44 surface expression through physical association with the AP-2 clathrin adaptor complex, which targets cell surface receptors for endocytosis through clathrin-coated vesicles (17). The μ 2 subunit of AP-2 is known to associate with

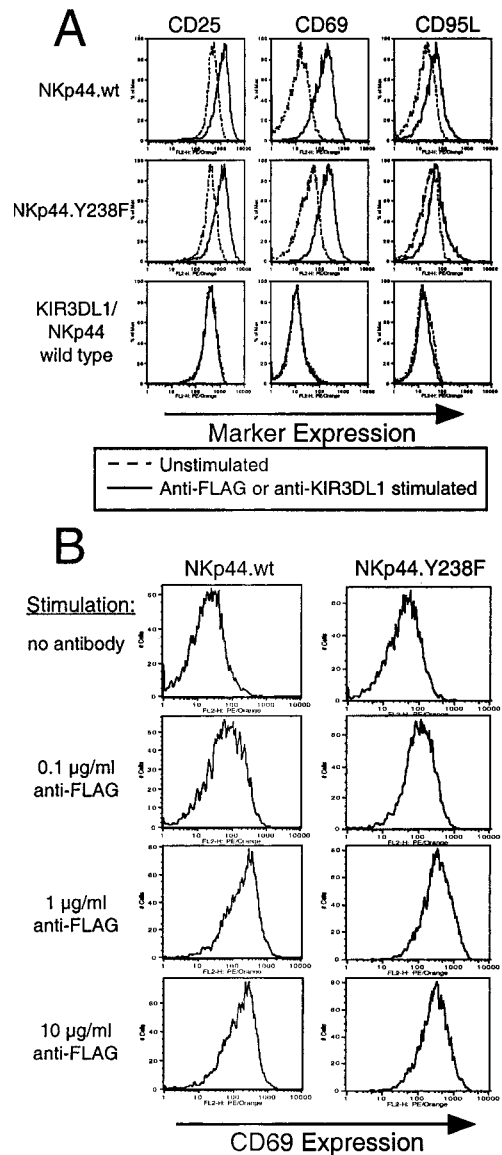


FIGURE 5. Up-regulation of activation markers by NKp44 engagement is not affected by the cytoplasmic tyrosine. **A**, Receptor-transduced NK-92 cells were cultured for 18 h in six-well plates (2 million/well) that had been precoated with anti-FLAG mAb (M2 for FLAG-NKp44 receptors) or mouse anti-3DL1 mAb (DX9 for 3DL1.p44.wt). Cells were harvested and stained with PE-conjugated Abs specific for CD25 or CD69 or with biotinylated anti-CD95L plus PE-conjugated streptavidin. Dashed lines are staining of unstimulated cells, and solid lines are staining of stimulated cells. **B**, CD69 expression in response to engagement of NKp44 with increasing concentrations of anti-FLAG mAb. NK-92 cells transduced with NKp44 receptors were cultured for 18 h in six-well plates (2 million/well) that had been precoated with anti-FLAG mAb (M2) at indicated concentrations. Cells were then harvested and stained with PE-conjugated anti-CD69 mAb.

Yxx ϕ sequences (ϕ = large hydrophobic or aromatic residue), which matches the ITIM-like sequence of NKp44 (18–20). Therefore, we used the yeast two-hybrid system to test whether the ITIM-like sequence of NKp44 acts as a tyrosine-based internalization motif through association with the μ 2 subunit (also known as AP50) of the AP-2 complex. As shown in Fig. 7, we did not detect any μ 2 association with the cytoplasmic domains of NKp44, KIR2DL1, or ICAM3, while interaction with the cytoplasmic domain of CD5 was readily detectable, as previously reported (15).

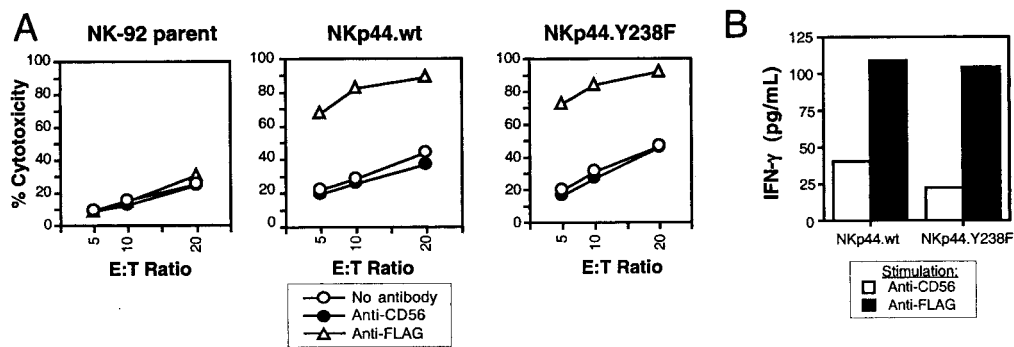


FIGURE 6. Cytotoxicity or IFN- γ production responses stimulated by NKp44 engagement are not influenced by the cytoplasmic tyrosine. *A*, Full-length FLAG-tagged NKp44 receptors (wt or Y238F) on transduced NK-92 cells were engaged with anti-FLAG mAb (M2; Δ), anti-CD56 mAb (B159; \bullet), or without added mAb (\circ) during a 4-h redirected cytotoxicity assay toward P815 target cells. NK-92 cells were assayed under resting conditions (day 4 after IL-2). *B*, NK-92 cells expressing FLAG-NKp44 receptors were stimulated for 18 h with plate-bound anti-FLAG mAb (\blacksquare) or anti-CD56 mAb (\square). Supernatants were collected and assayed for IFN- γ production by ELISA.

In accordance with this result, we did not detect differential internalization rates of NKp44.wt, NKp44.Y238F, and NKp44.trunc receptors in response to engagement with anti-FLAG mAb and secondary cross-linking Abs in NK-92 cells (data not shown). The truncated receptor (NKp44.trunc) was prepared, because AP-2 is also known to interact with phenylalanine motifs (Fxx ϕ) (21). Nonetheless, even the receptor lacking the majority of its cytoplasmic domain did not exhibit altered rates of internalization, recycling, or functional response (cytotoxicity or IFN- γ production). Taken together, our experiments indicate that the ITIM-like motif of NKp44 does not contribute to receptor signaling or internalization functions of the NKp44/DAP12 receptor complex.

Discussion

We found that surface expression of NKp44 requires DAP12 association, and the physical linkage to DAP12 is mediated via the transmembrane lysine (K183) on NKp44. As with other activating receptors on NK cells and T cells, the basis of the transmembrane association is most likely due to an ionic bond between this basic

amino acid on NKp44 and a corresponding aspartic acid residue in the transmembrane domain of DAP12 (22). Despite rigorous analysis, our studies indicate that an ITIM-like sequence in the cytoplasmic domain of NKp44 does not influence NK cell activating function or internalization of the receptor. Chimeric receptors were engineered to prove that the tyrosine-based sequence does not demonstrate inhibitory capacity or served as a docking site for the SH2-containing protein phosphatases, SHP-1, SHP-2, or SHIP. In addition, mutation of the cytoplasmic tyrosine to phenylalanine did not influence the activating function in the receptor complex as assessed by measuring the up-regulation of activation markers, cytotoxicity, or IFN- γ production. In summary, the noncovalently associated DAP12 appears to contribute all activating properties to NKp44.

It is indeed possible that the tyrosine-based sequence of NKp44 provides a functional role or roles that were not identified in our experiments. However, the lack of detectable recruitment of inhibitory phosphatases, despite strong tyrosine phosphorylation, and the lack of negative impacts upon several prominent functional responses triggered by NKp44, suggests that this role is subtle at best. Importantly, we also did not observe any evidence of a role for the tyrosine-based sequence in promoting activation through NKp44. Interestingly, we have not been able to convincingly demonstrate tyrosine phosphorylation of NKp44 upon treatment of NK-92 cells or primary NK cells with pervanadate. The fact that we observed tyrosine phosphorylation of the cytoplasmic domain in the context of the 3DL1.p44.wt chimeric receptor indicates that the tyrosine motif has the potential to serve as a kinase substrate as we had predicted, but the conditions that might result in such a phosphorylation event in the NKp44/DAP12 receptor complex are unclear. It is possible that the tyrosine is inaccessible in the receptor complex, due to physical obstruction by the DAP12 accessory protein.

We have found no evidence of a role for the tyrosine-based cytoplasmic sequence in promoting constitutive or ligation-induced internalization of NKp44 (data not shown), suggesting that it does not couple the receptor to clathrin adaptor complexes to mediate endocytosis through clathrin-coated pits. Furthermore, our yeast two-hybrid experiment demonstrated that the μ 2 subunit of the AP-2 clathrin adaptor did not interact with the NKp44 cytoplasmic domain. The μ 2 subunit is known to bind to Yxx ϕ motifs in cytoplasmic domains of transmembrane proteins, such as CD5 (15, 18–20), which served as a positive control in our experiments.

It is important to note that we did not clone any NKp44 cDNAs with alternative cytoplasmic domains. We originally embarked

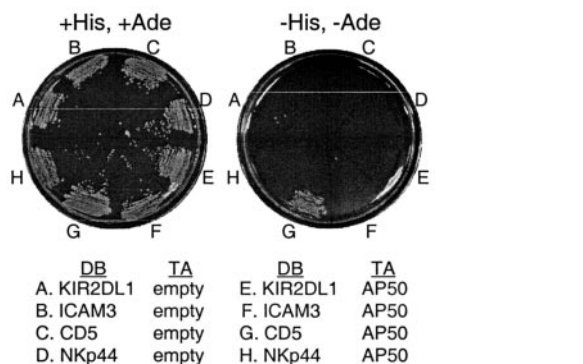


FIGURE 7. The AP-2 clathrin adaptor component, AP50 (μ 2), does not interact with the NKp44 cytoplasmic domain in the yeast two-hybrid system. The cytoplasmic domains of NKp44, KIR2DL1, ICAM3, and CD5 were each fused to the Gal4 DNA binding domain (DB) and expressed in a reporter yeast strain (AH109) in combination with the Gal4 transcriptional activation domain (TA) either alone (empty) or directly fused with the μ 2 subunit of AP-2, AP50. Transfected cells were streaked on agarose plates (lettered quadrants) under growth reporter selection conditions lacking histidine and adenine (-His, -Ade) or under nonrestrictive conditions in the presence of those amino acids (+His, +Ade). Growth under selecting conditions indicates a direct interaction between cytoplasmic domain and AP50, which was evident for the positive control, CD5.

upon these experiments based in part upon GenBank entries that described cDNAs with cytoplasmic frame-shifted sequences that encode proteins lacking the ITIM-like sequence (NKp44RG1 and NKp44RG2). Although we cannot rule out the existence of similar frame-shifted gene products in the human population, our data provide evidence that they would not appear to disrupt or enhance NKp44 function.

It has previously been proposed that ITIM-like sequences that were noted within the cytoplasmic domains of numerous activating receptors may directly impact upon their functions (23). Our work suggests that at least some of these sequences are not functional ITIMs. In addition, despite a great deal of analysis through mutations and sequence comparisons over the years, the consensus ITIM sequence remains loosely defined, and the specific primary sequence elements that constitute docking sites for distinct phosphatases (SHP-1, SHP-2, SHIP-1, and SHIP-2) continue to remain elusive. Another NK cell-activating receptor, KIR2DL4, also contains a cytoplasmic ITIM with proven inhibitory potential in combination with a basic transmembrane residue that enables activating function (11, 24). Interestingly, that ITIM does not seem to suppress or enhance NK cell activation through KIR2DL4, despite its strong inhibitory potential (24, 27). Alternatively, SHP-2 recruitment to ITIM-like sequences on several other activating receptors has been shown to potentiate activating function by SHP-2 serving as an adaptor molecule, as opposed to attenuation of the signaling via protein tyrosine phosphatase activity (25, 26). Therefore, ITIM or ITIM-like sequences on activating receptors must be analyzed on an individual basis to directly assess their impacts on function. In the case of NKp44, our evidence in this report indicates that the tyrosine-based cytoplasmic sequence is not an ITIM and does not attenuate or enhance activating function.

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