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Association of DAP12 with Activating CD94/NKG2C NK Cell Receptors

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Summary

While the inhibitory NK cell receptors for MHC class I express immunoreceptor tyrosine-based inhibitory motifs that recruit intracellular tyrosine phosphatases and prevent NK cell effector function, the activating NK cell receptors lack intrinsic sequences required for cellular stimulation. CD94/NKG2C, an activating NK cell receptor of the C-type lectin superfamily that binds to HLA-E, noncovalently associates with DAP12, a membrane receptor containing an immunoreceptor tyrosine-based activating motif. Efficient expression of CD94/NKG2C on the cell surface requires the presence of DAP12, and charged residues in the transmembrane domains of DAP12 and NKG2C are necessary for this interaction. These results provide a molecular basis for the assembly of NK cell receptors for MHC class I involved in cellular activation and inhibition.

Introduction

NK cells are lymphocytes that participate in innate immune responses against certain bacteria, parasites, and viruses (reviewed in Trinchieri, 1989; Scott and Trinchieri, 1995). How NK cells recognize pathogens is unclear; however, one aspect of this process may involve the detection and elimination of host cells that have lost or down-regulated expression of MHC class I as a consequence of infection. NK cells express receptors for MHC class I that can either activate or inhibit cell-mediated cytotoxicity and cytokine production (reviewed in Lanier, 1998a, 1998b). Several types of NK cell receptors for MHC class I have been identified (Lanier, 1998a). In humans, the killer cell inhibitory receptors (KIR) comprise a small family of molecules encoded by genes of the Ig superfamily (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Wagtmann et al., 1995a). Within the KIR family, certain isoforms possess two Ig domains (KIR2D) or three Ig domains (KIR3D) in the extracellular region that are involved in recognition of polymorphic HLA-C or HLA-B ligands, respectively (Litwin et al., 1994; Wagtmann et al., 1995b; Dohring and Colonna, 1996; Fan et al., 1996; Rajagopalan and Long, 1997; Rojo et al., 1997). Heterogeneity also exists in the transmembrane and cytoplasmic domains of different KIR molecules. Upon ligand binding, KIR having immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic domain (designated KIR2DL and KIR3DL) recruit SHP-1 and prevent NK cell effector function (Burshtyn et al., 1996; Campbell et al., 1996; Fry et al., 1996; Olcese et al., 1996). In contrast, KIR isoforms lacking ITIM and having a basic K amino acid in the transmembrane (KIR2DS and KIR3DS) have been implicated in NK cell activation (Biassoni et al., 1996; Olcese et al., 1997). KIR2DS are noncovalently associated with an immunoreceptor tyrosine-based activating motif (ITAM)–bearing adaptor molecule, DAP12, that is expressed on the surface of NK cells as a disulfide-bonded homodimer (Campbell et al., 1998; Lanier et al., 1998; Olcese et al., 1997). Upon cross-linking of KIR2DS, tyrosine residues in the ITAM of DAP12 become phosphorylated and recruit ZAP-70 or Syk, resulting in cellular activation (Lanier et al., 1998). Human *DAP12* is present on human chromosome 19q13.1 (Lanier et al., 1998) near the *KIR* gene family (Baker et al., 1995), demonstrating a genetic linkage between *KIR* and *DAP12*.

Another type of NK cell receptor, CD94/NKG2, is a heterodimer composed of an invariant CD94 glycoprotein that is disulfide-bonded to either an NKG2A or an NKG2C glycoprotein (Lazetic et al., 1996; Brooks et al., 1997; Carretero et al., 1997). *CD94* (Chang et al., 1995) and four NKG2 genes (*NKG2A*, *NKG2C*, *NKG2E*, and *NKG2D/F*) (Houchins et al., 1991; Plougastel and Trowsdale, 1997) are all members of the C-type lectin superfamily and are closely linked on human chromosome 12p12–p13 in the "NK complex" (Renedo et al., 1997). Rodent homologs of the human *CD94* and *NKG2* genes are located in the "NK complex" on mouse and rat chromosomes syntenic with human chromosome 12 (Dissen et al., 1997; Vance et al., 1997; Berg et al., 1998).

Antibodies against CD94 can either activate or inhibit NK cell–mediated cytotoxicity against Fc receptor–bearing targets, and different NK cell clones isolated from a single individual demonstrate heterogeneous behavior in these functional assays (Brumbaugh et al., 1996; Pérez-Villar et al., 1995, 1996). This phenomenon was explained by the finding that CD94 forms disulfide-linked heterodimers with either NKG2A or NKG2C (Lazetic et al., 1996; Brooks et al., 1997; Carretero et al., 1997; Cantoni et al., 1998). NKG2A contains an ITIM sequence in the cytoplasmic domain that upon receptor ligation becomes tyrosine-phosphorylated and recruits SHP-1 or SHP-2, which in turn inhibit NK effector function (Houchins et al., 1997; Le Drean et al., 1998). In contrast, NKG2C lacks an ITIM and receptor ligation results in NK cell activation (Houchins et al., 1997; Cantoni et al., 1998). CD94 is necessary to transport both NKG2A and NKG2C to the cell surface (Lazetic et al., 1996). Within the NK cell population in an individual, CD94/NKG2A and CD94/NKG2C receptors are expressed on overlapping subpopulations, and some NK cells may express CD94 proteins that are not associated with either NKG2A or NKG2C (Cantoni et al., 1998). Thus, CD94 and the NKG2 proteins can form a diverse receptor repertoire in an individual. CD94/NKG2A and CD94/NKG2C receptors recognize HLA-E (Borrego et al., 1998; Braud et al., 1998; Lee et al., 1998b), a nonclassical MHC class

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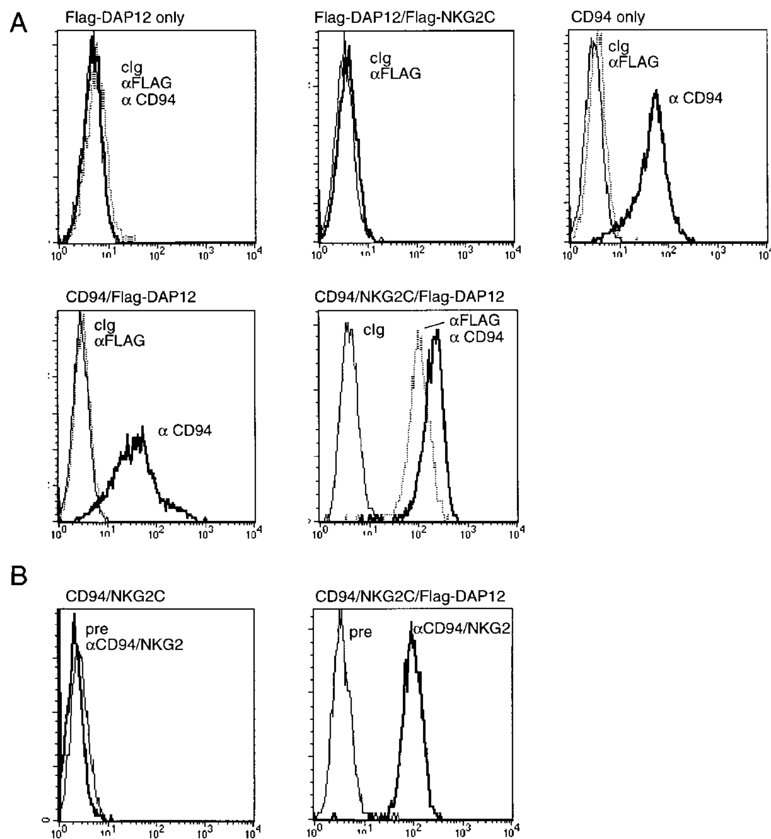

Figure 1. Expression of CD94, NKG2, and DAP12 on Ba/F3 Transfectants

Ba/F3 stably expressing the indicated receptors were stained with: (A) control mouse IgG1 (cIg), anti-CD94 mAb, or anti-Flag mAb (to detect Flag-DAP12 or Flag-NKG2C), or (B) rabbit anti-CD94/NKG2 antiserum or the prebleed rabbit serum (pre). Cells were analyzed by flow cytometry and data are presented as histograms (x axis, fluorescence, 4 decade log scale; y axis, number of cells). Because a mAb specific for NKG2C was not available for certain experiments, a Flag epitope was placed on the COOH terminus of the NKG2C protein (as indicated) to permit detection on the cell surface by immunofluorescence and in the cytoplasm by Western blot analysis.

I molecule that has the unique property of binding 9 amino acid peptides derived from the leader segments of other classical HLA class I proteins (Braud et al., 1997; Lee et al., 1998a). While the ITIM in NKG2A explains the inhibitory function of the CD94/NKG2A receptor, neither CD94 nor NKG2C possess sequences in their cytoplasmic domains that provide for intrinsic signaling capacity. However, the existence of a basic amino acid in the transmembrane of NKG2C suggested possible interactions with the DAP12 receptor.

Results and Discussion

Association of DAP12 with CD94/NKG2C Receptors

To determine whether DAP12 might be associated with the activating CD94/NKG2C receptor complex, we coinfected a mouse pre-B cell line, Ba/F3, with ecotropic retroviruses encoding human CD94, NKG2C, and DAP12 (containing a Flag epitope on the N terminus to permit detection on the cell surface). Consistent with prior results (Lanier et al., 1998), transfection of Flag-DAP12 alone into Ba/F3 cells does not permit cell surface expression of this receptor (Figure 1A), although Flag-DAP12 proteins were detected in the cytoplasm of these transfectants as determined by cytoplasmic staining and Western blot analysis (not shown). Similarly, we could not detect cell surface expression of NKG2C alone in Ba/F3 cells (not shown) or in Flag-DAP12$^+$ Ba/F3 transfectants coinfected with NKG2C (Figure 1A). In contrast, CD94 alone was expressed on the cell surface of Ba/F3 cells (Figure 1A). However, CD94 is not competent to transport Flag-DAP12 to the cell surface in Ba/F3 cells coinfected with both CD94 and Flag-DAP12 (Figure 1A), although Flag-DAP12 was detected in the cytoplasm of these transfectants by Western blot and cytoplasmic immunofluorescence (not shown). Furthermore, when CD94$^+$ Ba/F3 cells were infected with a retrovirus encoding NKG2C (Figure 1B), we were unable to detect CD94/NKG2C heterodimers on the cell surface, using an antiserum that detects the CD94/NKG2C complex (Lazetic et al., 1996; Braud et al., 1998) (although it is possible to obtain low levels of surface expression of CD94/NKG2C heterodimers using episomal vectors containing strong promoters in highly efficient transfection systems such as 293T cells [Lazetic et al., 1996; Braud et al., 1998]). When Ba/F3 cells were infected with retroviruses encoding human CD94, NKG2C, and Flag-DAP12, we detected expression of Flag-DAP12 (Figure 1A) and a CD94/NKG2C receptor (Figure 1B) on the cell surface of the CD94/NKG2C/DAP12 transfectants. Collectively, these experiments support the existence of a multisubunit receptor complex composed of CD94, NKG2C, and DAP12.

Ba/F3 transfectants expressing CD94, NKG2C, and Flag-DAP12 were labeled with ^{125}I, solubilized in digitonin detergent to preserve noncovalent membrane receptor complexes (Lanier et al., 1989), and immunoprecipitated with antibodies against human CD94 or Flag. As shown in Figure 2, immunoprecipitation with anti-CD94 from the CD94/NKG2C/Flag-DAP12 Ba/F3 transfectants revealed ^{125}I-labeled proteins consistent with the predicted mobility of NKG2C and Flag-DAP12. We previously reported that human CD94 does not label efficiently with ^{125}I (Lazetic et al., 1996; Phillips et al., 1996),

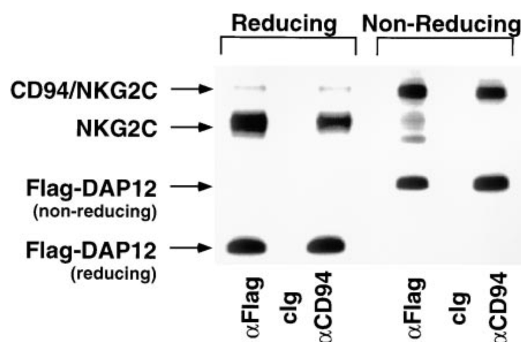

Figure 2. Coimmunoprecipitation of DAP12 and CD94/NKG2C

Ba/F3 cells stably expressing CD94/NKG2C/Flag-DAP12 receptors were ^{125}I labeled and lysed in digitonin detergent, and antigens were immunoprecipitated with cIg, anti-Flag mAb, or anti-CD94. Samples were analyzed by SDS–PAGE in reducing or nonreducing conditions. The CD94/NKG2C heterodimer migrates at ~70 kDa under nonreducing conditions, and the ^{125}I-labeled NKG2C glycoprotein migrates at ~40 kDa in reducing conditions. As previously reported (Lazetic et al., 1996; Phillips et al., 1996), CD94 does not ^{125}I-label. DAP12 migrates as a disulfide-bonded homodimer in nonreducing conditions.

so the ~40 kDa radiolabeled subunit immunoprecipitated with anti-CD94 mAb represents an NKG2C glycoprotein that is disulfide-bonded to CD94 (Lazetic et al., 1996). When analyzed using nonreducing conditions, Flag-DAP12 migrated predominately as a disulfide-bonded homodimer and the mobility of NKG2C was consistent with the existence of a CD94/NKG2C heterodimer. Therefore, it appears that the minimal CD94/NKG2C-DAP12 receptor complex may be a tetramer comprised of a disulfide-linked DAP12 homodimer noncovalently associated with a disulfide-linked CD94/NKG2C heterodimer. Further studies are required to establish the stoichiometry of the native complex on the cell surface.

The Role of Charged Amino Acids in the Transmembrane of KIR, NKG2, and DAP12 Receptors in the Assembly of the Multisubunit Complexes

The NKG2A and NKG2C proteins demonstrate 75% amino acid identify (Houchins et al., 1991), and both CD94/NKG2A and CD94/NKG2C receptors bind to a common ligand, HLA-E (Braud et al., 1998). A conspicuous difference between NKG2A and NKG2C is the presence of a basic residue in the transmembrane of NKG2C that is absent in NKG2A and CD94 (Figure 3). In contrast to NKG2C, infection of CD94^{+} Ba/F3 cells with a retrovirus encoding human NKG2A permits expression of a CD94/NKG2A complex on the cell surface in the absence of DAP12 (Figure 4A). The presence of a CD94/NKG2A complex on Ba/F3 cells does not permit expression of Flag-DAP12 on the cell surface (Figure 4A), although Flag-DAP12 proteins were detected in the cytoplasm of these transfectants by immunofluorescence and Western blot analysis (not shown).

Because other multisubunit membrane receptors have been shown to associate via salt bridges formed by acidic and basic amino acids in their transmembranes (e.g., CD3/TcR [Morley et al., 1988; Bonifacino et al., 1991; Cosson et al., 1991]), we examined the requirement of the D residue in DAP12 for association with CD94/NKG2C. The D residue in Flag-DAP12 was converted to A by site-directed mutagenesis, and this mutant receptor was transfected into Ba/F3 cells. Unlike wild-type Flag-DAP12 (Figure 1A), the D-A transmembrane Flag-DAP12 mutant receptor was expressed on the cell surface in the absence of other subunits (Figure 4B), indicating that the D residue in the transmembrane serves as a retention signal for DAP12, similar to the function of the charged residues in the transmembrane of the CD3 proteins (Bonifacino et al., 1990, 1991; Cosson et al., 1991). As noted previously, Ba/F3 cells transfected with CD94 and NKG2C do not efficiently express a CD94/NKG2C heterodimer on the cell surface in the absence of DAP12 (Figure 1B). Infection of these CD94/NKG2C^{+} Ba/F3 transfectants with the D-A transmembrane Flag-DAP12 mutant receptor did not permit efficient expression of CD94/NKG2C on the cell surface (Figure 4B), as indicated by the marginal reactivity of these cells with an anti-CD94/NKG2 specific antisera (although NKG2C proteins were detected in the cytoplasm of the transfectant by Western blot analysis; not shown).

CD94 Transmembrane	LISGTLGIICLSLMATLGILL	extracellular
NKG2A Transmembrane	LIVGILGIICLILMASVVTIVVI	extracellular
NKG2C Transmembrane	VLGIICIVLMATVL**K**TIVLIPF	extracellular
DAP12 Transmembrane	VLAGIVMG**D**LVLTVLIALAVYFL	cytoplasmic
KIR2DS2 Transmembrane	LIGTSVV**K**IPFTILLFFLL	cytoplasmic
KIR2DL2 Transmembrane	LIGTSVVIILFILLLFFLL	cytoplasmic

Figure 3. Transmembranes of CD94, NKG2, DAP12, and KIR Proteins

The consensus transmembrane segments of DAP12, CD94, NKG2A, NKG2C, KIR2DL2, and KIR2DS2 were predicted based on analysis of the protein structures using the SOSUI program, the DAS membrane predictor server, and the TMPred program. CD94, NKG2A, and NKG2C are type II proteins, whereas DAP12, KIR2DL2, and KIR2DS2 are type I proteins. Locations of the cytoplasmic or extracellular domains on the COOH side of the transmembrane segments are indicated. Charged amino acids in the transmembrane are bold.

Comparison of the transmembrane domains of NKG2A and NKG2C indicates the presence of a K residue in NKG2C, suggesting that this residue may be responsible for interaction with the D residue in DAP12 (Figure 3). Therefore, the K in NKG2C was converted to L by site-directed mutagenesis and the K-L transmembrane NKG2C mutant was transfected into Ba/F3 cells expressing DAP12 and CD94. As shown in Figure 4C, Ba/F3 cells cotransfected with CD94, and the K-L transmembrane NKG2C mutant receptor did not permit surface expression of Flag-DAP12, although DAP12 was detected in the cytoplasm by Western blot analysis (not shown). Very low levels of a CD94/K-L transmembrane NKG2C mutant receptor were detected on the surface of these transfectants using an anti-CD94/NKG2C antiserum (Figure 4C). Although the K residue in the transmembrane of NKG2C might serve as a retention signal, it should be noted that NKG2C also expresses the motif DxxxLL that is also present in CD3γ and has been implicated in the degradation, transport, and localization of CD3 proteins (Letourneur and Klausner, 1992; Dietrich et al., 1994, 1996, 1997) and in the binding of adaptor protein 1 (AP-1) and adaptor protein 2 (AP-2) (Dietrich et al., 1997).

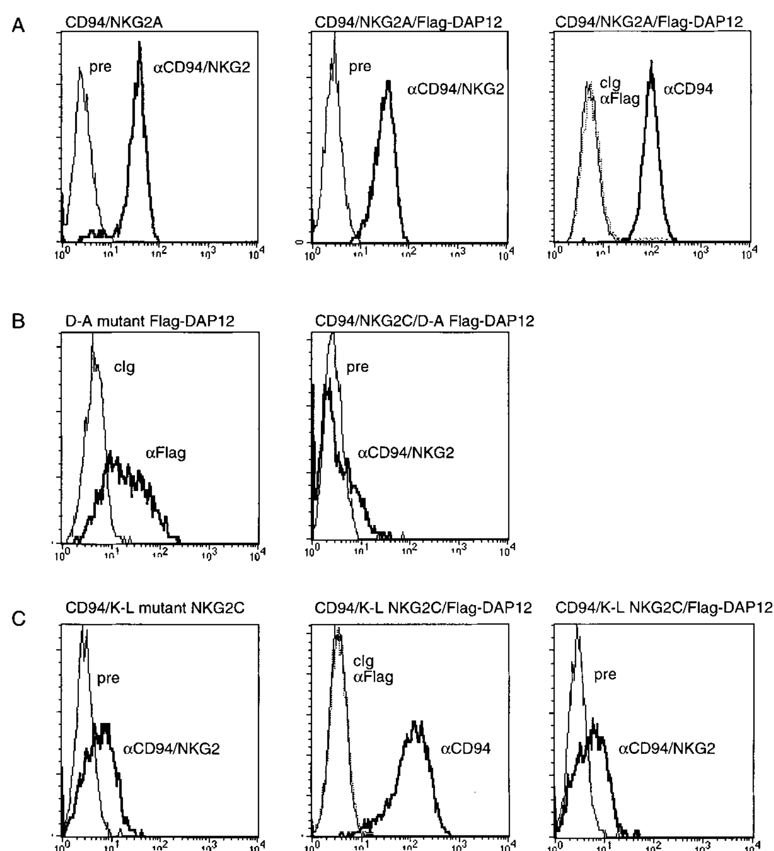

Figure 4. Expression of Transmembrane Mutants of DAP12 and NKG2C

Ba/F3 cells stably expressing CD94/NKG2A, CD94/NKG2A/Flag-DAP12, the D-A transmembrane NKG2C mutant, CD94/NKG2C/D-A transmembrane Flag-DAP12 mutant, CD94/K-L transmembrane NKG2C mutant, or CD94/K-L transmembrane NKG2C mutant/Flag-DAP12 receptors were stained with cIg, anti-CD94 mAb, anti-Flag mAb, rabbit anti-CD94/NKG2 antiserum, or the prebleed rabbit serum (pre), as indicated. Data are presented as in Figure 1.

Signal Transduction via CD94/NKG2C/DAP12 and KIR2DS2/DAP12 Complexes

Ligation of KIR2DS2 in transfectants expressing KIR2DS2/DAP12 complexes results in the tyrosine phosphorylation of DAP12 and other cellular substrates and the association of phosphorylated DAP12 with Syk (Lanier et al., 1998). Ligation of either CD94 or Flag-DAP12 on Ba/F3 transfectants expressing CD94/NKG2C/DAP12 complexes caused tyrosine phosphorylation of numerous cellular proteins (Figure 5A), including DAP12 (Figure 5B) and Syk (Figure 5C). These results indicate that cross-linking CD94/NKG2C induces cellular activation, presumably via DAP12. We were unable to address whether ligation of CD94/NKG2C in the absence of DAP12 or in transfectants expressing the D-A transmembrane Flag-DAP12 mutant has functional consequences because CD94/NKG2C was not efficiently expressed in the absence of wild-type DAP12.

Unlike CD94/NKG2C, KIR2DS2 molecules are expressed on the cell surface in the absence of DAP12, although they are unable to induce cellular activation (Bléry et al., 1997; Lanier et al., 1998). KIR2DS2^{+} Ba/F3 cells were infected with retroviruses encoding either wild-type Flag-DAP12 or the D-A transmembrane Flag-DAP12 mutant receptor. Both KIR2DS2 and the mutant DAP12 protein were expressed on the cell surface. However, the D-A transmembrane Flag-DAP12 mutant protein was not coimmunoprecipitated with KIR2DS2 from ^{125}I-labeled transfectants (Figure 6A). Furthermore, ligation with anti-KIR mAb failed to activate these cells, whereas direct cross-linking of the D-A transmembrane Flag-DAP12 mutant receptor with anti-Flag mAb did induce tyrosine phosphorylation of cellular proteins (Figure 6B). Like NKG2A and NKG2C, the KIR2DS2 protein has a counterpart, KIR2DL2, that lacks a charged amino acid in the transmembrane (Figure 3) and contains an ITIM in its cytoplasmic domain. We previously reported that KIR2DL2 is unable to associate with DAP12 (Lanier et al., 1998). Collectively, these findings indicate that the association of DAP12 with either KIR2DS2 or CD94/NKG2C complexes likely results from interactions involving the transmembrane domains of these proteins.

The stoichiometry of DAP12 and KIR2DS2 or CD94/NKG2C in these complexes has not been determined. A DAP12 disulfide-linked homodimer possesses two D residues (i.e., one in each DAP12 protein) that could interact with the K residues present in the transmembranes of KIR2DS2 or NKG2C (Figure 3). Because CD94 lacks charged residues in the transmembrane (Figure 3), DAP12 may be able to function as an adaptor permitting the association of two KIR2DS2 monomers or two CD94/NKG2C heterodimers with a single DAP12 homodimer. Further studies are required to address this possibility. This model would be advantageous for more efficient ligand binding and signal transduction.

Association of DAP12 and CD94 in Human NK Cells

CD94/NKG2C receptors previously have been implicated in NK cell activation (Houchins et al., 1997; Cantoni et al., 1998). An NK cell clone and a polyclonal NK cell line were selected based on their ability to mediate redirected cytotoxicity against the Fc receptor–bearing

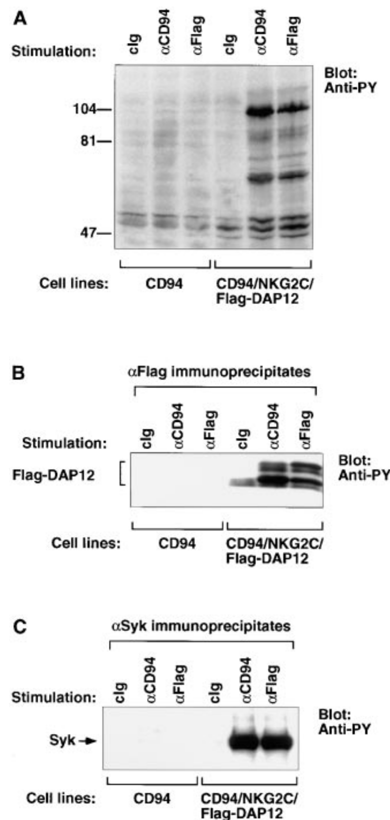

Figure 5. Stimulation of CD94/NKG2C/Flag-DAP12

Ba/F3 cells stably expressing CD94 only or CD94/NKG2C/Flag-DAP12 receptors were stimulated with cross-linked cIg, anti-CD94 mAb, or anti-Flag mAb, as indicated.

(A) Total cell lysates were resolved by SDS–PAGE (9% gels) and analyzed by Western blot using an anti-phosphotyrosine mAb.

(B and C) Flag-DAP12 (B) and Syk (C) proteins were immunoprecipitated and analyzed by Western blot using an anti-phosphotyrosine mAb, as indicated. The multiple bands seen in (B) represent different phosphorylation species of DAP12.

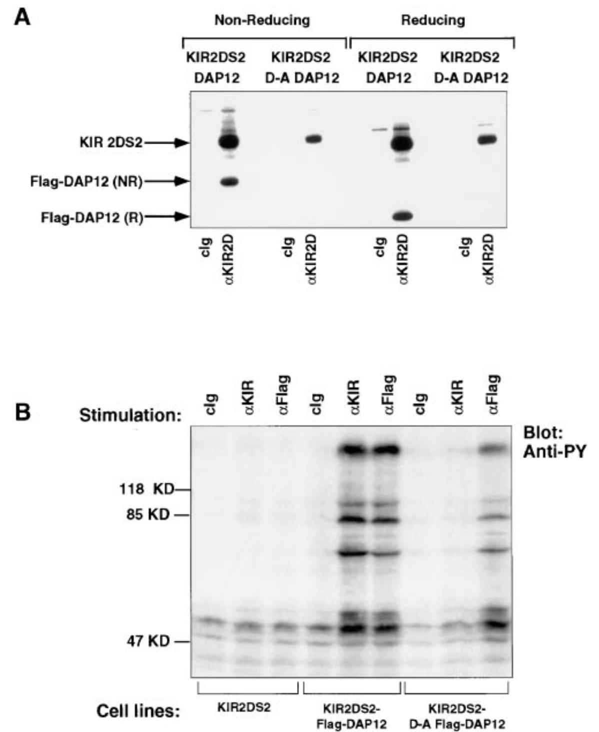

Figure 6. Coimmunoprecipitation of Wild-Type and Mutant KIR2DS2/Flag-DAP12 Receptors and Cellular Activation

(A) Ba/F3 cells stably expressing KIR2DS2/Flag-DAP12 receptors or KIR2DS2/D-A transmembrane Flag-DAP12 mutant receptors were ^{125}I labeled and lysed in digitonin detergent, and antigens were immunoprecipitated with cIg or anti-KIR2D mAb DX27. Samples were analyzed by SDS–PAGE in reducing or nonreducing conditions. ^{125}I proteins with migration characteristics of monomeric Flag-DAP12 ([R], reducing), dimeric Flag-DAP12 ([NR], nonreducing) and KIR2DS2 are indicated. Anti-Flag mAb immunoprecipitated the ^{125}I-labeled D-A transmembrane Flag-DAP12 mutant protein, but did not coimmunoprecipitate KIR2DS2 from the transfectant expressing both KIR2DS2 and the D-A transmembrane Flag-DAP12 mutant receptor (not shown).

(B) Ba/F3 cells stably expressing KIR2DS2 only, KIR2DS2/Flag-DAP12, or KIR2DS2/D-A transmembrane Flag-DAP12 mutant receptors were stimulated with cross-linked cIg, anti-KIR2DS2 mAb DX27, or anti-Flag mAb, as indicated. Total cell lysates were resolved by SDS–PAGE (9% gels) and analyzed by Western blot using an anti-phosphotyrosine mAb.

P815 target cell in the presence of anti-CD94 mAb (not shown), suggesting the presence of an activating CD94-associated receptor complex, probably CD94/NKG2C (Cantoni et al., 1998). The NK cell clone and the polyclonal NK cell line were ^{125}I labeled and lysed in digitonin detergent to preserve multisubunit receptor complexes, and DAP12-associated proteins were coimmunoprecipitated using an anti-DAP12 antiserum. DAP12-associated proteins were eluted with a pH 11.5 buffer to dissociate the complexes, and then the eluted proteins were reimmunoprecipitated with a control mAb or anti-CD94 mAb. As shown in Figure 7 for the polyclonal NK cell line, anti-CD94 mAb specifically reacted with a ^{125}I protein eluted from the initial anti-DAP12 immunoprecipitate. On SDS–PAGE analysis, this molecule migrated at $\sim$70 kDa in nonreducing conditions and $\sim$40 kDa in reducing conditions. Equivalent results were obtained using the NK cell clone (not shown). Because CD94 itself does not ^{125}I label (Lazetic et al., 1996; Phillips et al., 1996), it seems likely that the CD94-associated ^{125}I-labeled protein represents NKG2C, although NKG2C-specific serological reagents are not available to confirm this. Nonetheless, these finding demonstrate the existence of a CD94/DAP12 receptor complex on the cell surface of human NK cells.

Paired Activating and Inhibitory Receptors

The KIR gene family encodes receptors that have been implicated in either cellular activation or inhibition (Biassoni et al., 1996; Olcese et al., 1997). The inhibitory receptors contain ITIM sequences in their cytoplasmic domains and lack charged residues in the transmembrane segments, whereas the activating receptors lack ITIM, often have shorter cytoplasmic regions, and possess a charged amino acid in the transmembrane. This general strategy is also evident in the NKG2 (Houchins et al., 1991), Ly49 (Smith et al., 1994), PIR (Hayami et al., 1997; Kubagawa et al., 1997), and ILT (LIR) (Borges et al., 1997; Samaridis and Colonna, 1997) gene families,

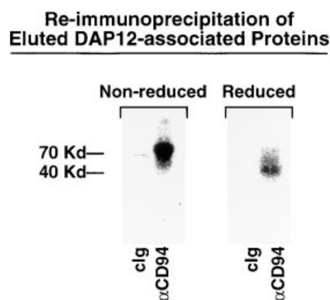

Figure 7. Association of CD94 and DAP12 in Human NK Cells

A human polyclonal NK cell line was ^{125}I labeled and lysed in digitonin detergent, and DAP12-associated antigens were immunoprecipitated with affinity-purified rabbit anti-human DAP12 antiserum. DAP12-associated proteins were eluted in 50 mM diethylamine (pH 11.5), the eluates were neutralized in 1% NP-40 lysis buffer, and DAP12-eluted proteins were reimmunoprecipitated with anti-CD94 mAb–coupled Sepharose beads or anti-NKR-P1A mAb–coupled Sepharose beads (used as a negative control (cIg) to demonstrate the specific interaction between DAP12 and the CD94 receptor complex). NKR-P1A was used as a specificity control because prior studies demonstrated that while these NK cells express NKR-P1A this molecule is not associated with DAP12 as determined by cotransfection experiments (our unpublished data). Samples were analyzed by SDS–PAGE in reducing and nonreducing conditions. The DAP12-associated protein reimmunoprecipitated with anti-CD94 mAb migrated at ~70 kDa under nonreducing conditions and ~40 kDa in reducing conditions. As previously reported (Lazetic et al., 1996; Phillips et al., 1996), CD94 does not ^{125}I-label.

which all include potentially inhibitory and activating receptors.

We have shown that DAP12 associates with the activating isoforms of both the KIR and CD94/NKG2 receptors. The inhibitory CD94/NKG2A and activating CD94/NKG2C receptors both bind the same ligand, HLA-E (Braud et al., 1998). What is the biological rationale for paired inhibitory and activating receptors recognizing MHC class I? The activating CD94/NKG2C/DAP12 receptor complex may function to stimulate tyrosine kinases that phosphorylate the ITIM sequences in the inhibitory NKG2A receptor, resulting in the recruitment of SHP-1 or SHP-2 (Le Drean et al., 1998). However, this seems unlike since NKG2A and NKG2C are differentially expressed within the total NK cell population and only a subset of NK cells expresses both receptors (Houchins et al., 1997; Cantoni et al., 1998). The existence of NK cells expressing CD94/NKG2C, in the absence of the inhibitory CD94/NKG2A receptor, provides the potential for activation of these cells upon encountering HLA-E. HLA-E is broadly expressed in normal tissues (Geraghty et al., 1992; Ulbrecht et al., 1992; Lee et al., 1998a); therefore, activation of NK cells via CD94/NKG2C/DAP12 might result in autoimmunity. However, recent studies suggest that all NK cell clones appear to express at least one inhibitory receptor (either a KIR or CD94/NKG2A) against a self MHC class I ligand, thus preventing destruction of normal autologous tissues (Uhrberg et al., 1997; Valiante et al., 1997). NK cell clones expressing activating CD94/NKG2C/DAP12 receptors and an inhibitory KIR against a self class I ligand could potentially recognize and eliminate host cells that have lost expression of the KIR class I ligand, but retained expression of HLA-E. This model requires experimental testing, but would provide defense against pathogens that encode leader peptides competent to bind HLA-E, but down-regulate expression of conventional MHC class I molecules as a consequence of infection (Lanier, 1998a).

Experimental Procedures

Transfectants

cDNAs used were human *CD94* (Chang et al., 1995), *NKG2A* (Houchins et al., 1991), *NKG2C* (Houchins et al., 1991), *KIR2DS2* (NKAT5 [Colonna and Samaridis, 1995]), and *Flag-DAP12* (Lanier et al., 1998). The D-A transmembrane *Flag-DAP12* mutant cDNA with an A residue (codon GCC) substituted for the D residue (codon GAC) and the K-L transmembrane *NKG2C* mutant cDNA with an L residue (TTA) substituted for K (codon AAA) were generated by PCR mutagenesis using conventional techniques. An *NKG2C* cDNA containing a Flag epitope on the COOH terminus immediately prior to the *NKG2C* stop codon was generated by PCR. cDNAs were sequenced and subcloned into the pMX-neo or pMX-puro retroviral vectors (Onihsi et al., 1996). Plasmid DNA was transfected into F-NX-E ecotropic retrovirus packaging cells (a generous gift from G. Nolan, Stanford University) using lipofectamine (GIBCO-BRL) (Onihsi et al., 1996). Viral supernatants were collected two days later and used to infect mouse Ba/F3 pre-B cells (Onihsi et al., 1996). Two days postinfection, cells were switched to selection medium and Ba/F3 cells stably expressing human NK cell receptors were sorted by flow cytometry for homogeneous high level expression.

Antibodies and Flow Cytometry

mAbs used were anti-CD94 (DX22 [Phillips et al., 1996] or HP-3D9 mAb [Lopez-Botet, 1995]), anti-KIR2D mAb (DX27 [Phillips et al., 1996]), anti-NKR-P1A (DX1 [Lanier et al., 1994]), anti-Flag (M2 mAb, Kodak), anti-NKG2A/C (8E4 mAb [Houchins et al., 1997]), and control mouse IgG1 mAb (Becton Dickinson, San Jose, CA). Rabbit antiserum specific for the CD94/NKG2A and CD94/NKG2C heterodimers was prepared as described (Lazetic et al., 1996). FITC-conjugated goat anti-rabbit Ig and FITC-conjugated anti-mouse Ig second antibodies were purchased from CalTag (So. San Francisco, CA). Immunofluorescence and flow cytometry were performed as described (Lanier and Recktenwald, 1991).

Biochemistry

Transfected Ba/F3 cells were labeled with ^{125}I and solubilized in digitonin lysis buffer (pH 7.8, 1% digitonin, 0.12% Triton X-100, 150 mM NaCl, 20 mM triethanolamine, 0.01% NaN3, and protease inhibitors) (Lanier et al., 1989). Cell lysates were incubated on ice for 2 hr with Pansorbin (Calbiochem) coated with rabbit anti-mouse/rat Ig (Sigma) and anti-CD94 (DX22 mAb), anti-Flag (M2 mAb) or control IgG and then washed. Immunoprecipitates were resuspended in SDS–PAGE sample buffer in the presence or absence of 10% 2-mercaptoethanol, run on 18% Tris/glycine gels (Novex), and visualized by using a PhosphorImager (Molecular Dynamics).

A human NK cell clone and a polyclonal human NK cell line ($CD3^-$,$CD56^+$ peripheral blood NK cells cultured as described [Yssel et al., 1984]) were labeled with ^{125}I and solubilized in digitonin lysis buffer. ^{125}I cell lysates were precleared overnight with Pansorbin coated with rabbit Ig and then incubated on ice for 2 hr with Pansorbin coated with an affinity-purified rabbit anti-DAP12 antiserum (generated by standard methods against a GST fusion protein containing the entire cytoplasmic domain of human DAP12). DAP12-associated proteins were eluted in 25 μl of 50 mM diethylamine (pH 11.5) and transferred to 0.5 ml 1% NP-40 lysis buffer (50 mM Tris, 150 mM NaCl [pH 8.0] containing protease inhibitors) with 10 mg/ml BSA carrier protein. The DAP12-associated eluted proteins were reimmunoprecipitated with anti-CD94 mAb (HP-3D9 and DX22)–coupled Sepharose beads or anti-NKR-P1A mAb (DX1)–coupled Sepharose beads (used as a negative control). Immunoprecipitates were washed in 1% NP-40 lysis buffer, resuspended in SDS–PAGE

sample buffer in the presence or absence of 10% 2-mercaptoethanol, run on 18% Tris/glycine gels, and visualized by using a PhosphorImager.

Western blot analysis using anti-Flag (M2 mAb) or anti-NKG2A/C (8E4 mAb [Houchins et al., 1997]) was performed as described (Phillips et al., 1996). 8E4 mAb detects both NKG2A and NKG2C by Western blot analysis (Houchins et al., 1997), but does not immunoprecipitate or bind to these antigens in immunofluorescence assays.

Cellular Stimulation

Transfected Ba/F3 cells were suspended in cold PBS with 0.5% BSA at 5×10^7 cells/ml containing 20 μg/ml mAb recognizing CD94, Flag-DAP12, or KIR2DS2. Cells were incubated on ice for 30 min, washed, resuspended in 10 μg/ml goat anti-mouse IgG $F(ab')_2$ (Jackson Immunoresearch), and incubated for 3 min at 37°C. Cells were pelleted, resuspended at 10^8/ml in ice-cold lysis buffer (1% NP-40, 10 mM Tris [pH 7.4], 150 mM NaCl containing the protease and phosphate inhibitors aprotinin, leupeptin, PMSF, EDTA, $NaVO_4$, and NaF) as described (Lanier et al., 1998). Syk and Flag-DAP12 were immunoprecipitated with rabbit anti-Syk antiserum (generously provided by Joe Bolen, DNAX) or anti-Flag (M2 mAb). Cell lysates ($2-3 \times 10^6$ cell equivalents) and immunoprecipitates were run on Tris/glycine gels, blotted onto Immobilon membranes (Millipore), blocked, probed with horseradish peroxidase–conjugated anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology), washed, and developed with a chemiluminescent substrate (Pierce).

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