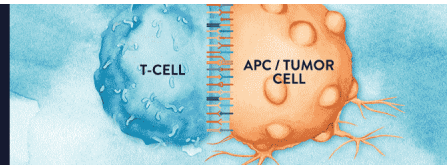


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Coligan

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NK Cell CD94/NKG2A Inhibitory Receptors Are Internalized and Recycle Independently of Inhibitory Signaling Processes

Francisco Borrego,¹ Juraj Kabat, Tolib B. Sanni, and John E. Coligan

Human CD94/NKG2A is an inhibitory receptor that recognizes HLA-E and is expressed by NK cells and a subset of T cells. We have analyzed the cellular trafficking of the CD94/NKG2A receptor using the NKL cell line and peripheral blood NK cells. Flow cytometric, confocal microscopic, and biochemical analyses show that CD94/NKG2A continuously recycles in an active process that requires the cytoskeleton between the cell surface and intracellular compartments that are distinguishable from recycling compartments used by well-characterized receptors, such as transferrin receptor (CD71). CD94/NKG2A, an inhibitory receptor, traffics differently from the closely related CD94/NKG2C molecule, an activating receptor. Using transfection/expression analyses of wild-type and mutant CD94/NKG2A molecules in the HLA-E negative rat basophilic cell line RBL-2H3, we demonstrate that CD94/NKG2A internalization is independent of ligand cross-linking or the presence of functional immunoreceptor tyrosine-based inhibition motifs. Thus, the mechanisms that control cell surface homeostasis of CD94/NKG2A are independent of functional signaling. *The Journal of Immunology*, 2002, 169: 6102–6111.

Natural killer cells play a role in protection against tumorigenesis and viral infections (1, 2). They express inhibitory receptors that inhibit cytotoxicity and cytokine production upon recognition of MHC class I molecules (3, 4). By this mechanism, NK cells detect the integrity of cells through their loss of expression of MHC class I protein, a feature known as the missing self-hypothesis (5). In humans, three distinct families of genes encoding inhibitory receptors for HLA class I molecules have been defined (4). The first family of receptors are type I transmembrane molecules belonging to the Ig superfamily and are called killer cell Ig-like receptors. The second group of receptors, also belonging to the Ig superfamily, named Ig-like transcripts, are expressed mainly on B, T, dendritic, and myeloid cells. However, some members of this group are also expressed on NK cells. The ligands for the killer cell Ig-like receptor and some of the Ig-like transcript receptors include classical (class Ia) HLA class I molecules, as well the nonclassical (class Ib) HLA-G molecule and the human CMV-encoded class I-like molecule UL18. The third family of HLA class I receptors are C-type lectin family members and are composed of heterodimers of CD94 covalently associated with a member of the NKG2 family of molecules (6, 7). The ligand for the inhibitory members of this family is the nonclassical class I molecule HLA-E (8–10).

NK inhibitory receptors share a common regulatory sequence of amino acids in their cytoplasmic tails, the immunoreceptor tyrosine-based inhibitory motifs (ITIMs).² Human NKG2A has two

ITIMs which contain tyrosine residues that are phosphorylated, presumably by a src tyrosine kinase, following interaction of CD94/NKG2A with HLA-E expressed on target cells. The phosphorylated ITIMs can recruit and activate the phosphatase Src homology 2 domain-bearing tyrosine phosphatase-1 (SHP-1), which suppresses the signal generated from cell surface-activating receptors (11, 12).

NK cells express a large variety of activating receptors that recognize ligands expressed by normal cells, as well as tumor and infected cells (13). Cross-linking of these receptors activates signaling cascades that result in cytokine production and killing of target cells. Under normal conditions, NK cells express at least one inhibitory receptor capable of interacting with autologous MHC class I molecules, which are a hallmark of normal somatic cells (14). The maintenance of adequate levels of inhibitory receptors on the cell surface of NK cells is required to suppress the constant stimulation of NK cells provided by the ligation of activating receptors (15).

A common feature of many cell surface receptors is their constitutive or ligand-induced endocytosis, and subsequent recycling back to the cell surface (16). CD71 (transferrin receptor), for example, is constitutively recycled through both peripheral/periplasmic and perinuclear recycling pathways (17). The pathways involved in the trafficking of some receptors in the immune system have been very well described. For example, despite a rapid rate of internalization in the absence of ligation, efficient recycling allows resting T cells to maintain stable levels of TCR expression (18–20). Following TCR ligation by MHC:peptide complexes, there is significant down-modulation of TCR-CD3 from the cell surface (21). Liu et al. (19) determined that this was not due to an accelerated rate of constitutive internalization, but instead to a retention of ligated complexes resulting in an alteration of TCR intracellular trafficking that leads to degradation by lysosomes and proteasomes. More recently, other authors have proposed that ligand-induced TCR internalization and down-regulation is a process independent of constitutive TCR cycling (20). The B cell receptor (BCR) also undergoes constitutive internalization (22). However,

Receptor Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852
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¹ Address correspondence and reprint requests to Dr. Francisco Borrego, Receptor Cell Biology Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Twinbrook II, Room 205, 12441 Parklawn Drive, Rockville, MD 20852. E-mail address: Fborrego@niaid.nih.gov

² Abbreviations used in this paper: ITIM, immunoreceptor tyrosine-based inhibition motif; BrefA, brefeldin A; CHX, cyclohexamide; ITAM, immunoreceptor tyrosine-based activation motif; Lata, latrunculin A; MESNA, 2-mercaptoethanesulfonic acid; MFI, mean fluorescence intensity; SHP-1, Src homology 2 domain-bearing tyrosine

phosphatase-1; BCR, B cell receptor; RBL-2H3 cell, rat basophilic leukemia cell; EGFP, enhanced green fluorescent protein.

after ligation there is an increase in the internalization and turnover rate of BCR and accelerated transport of the BCR to the MHC class II peptide-loading compartment (23).

The expression of functional NK inhibitory receptors must be ensured by tightly regulated bioprocessing pathways. The fact that NK cells have evolved to express several types of inhibitory receptors specific for MHC class I molecules seems to emphasize the importance of maintaining expression of such receptors. CD94/NKG2A appears to function as the failsafe inhibitory receptor because it is expressed on the vast majority of human NK cells and its ligand, HLA-E, is expressed on virtually all normal cells (24). The mechanisms controlling CD94/NKG2A cell surface expression on NK cells are unknown, as well as the processes that regulate cellular trafficking. In this report, we show that CD94/NKG2A is continuously recycled between the cell surface and cytoplasm, and that this recycling is independent of ligation and the transmission of inhibitory signals. Because CD94/NKG2A receptors are in the constant presence of ligand expressed by normal cells, the detachment of recycling from ligation/inhibitory processes likely facilitates the maintenance of a pool of CD94/NKG2A receptors on the cell surface available for interaction with HLA-E.

Materials and Methods

Cell lines and mAb

Polyclonal NK cells were isolated from peripheral blood using an NK cell isolation kit (Miltenyi Biotec, Auburn, CA). The purity of the isolated cell population was confirmed by flow cytometry. NK cells were cultured in IMDM (BioWhittaker, Walkersville, MD) supplemented with 500 U/ml of rIL-2 (Biological Resources Branch, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD), 10% human AB serum (BioWhittaker), and L-glutamine (BioSource International, Rockville, MD). Feeder autologous cells were added at days 0 and 5 of the culture. The NKL cell line was grown in RPMI 1640 (BioSource International) supplemented with L-glutamine, sodium pyruvate, 200 U/ml of rIL-2, and 10% of human AB serum. Rat basophilic leukemia cells (RBL-2H3 cells) were transfected with cDNAs for NKG2A or a double tyrosine mutant of NKG2A along with cDNA for CD94, as previously described (12). An NKG2A-EGFP fusion protein was expressed by cloning NKG2A cDNA lacking a stop codon into the *EcoRI/BglIII* sites of pEGFP-C1 (Clontech Laboratories, Palo Alto, CA). In this protein, the EGFP domain is attached to the N terminus of NKG2A, which is in the intracellular domain of the receptor. The mouse pre-B cell line Ba/F3 expressing CD94/NKG2C/DAP12 was kindly provided by J. H. Phillips (DNAX Research Institute, Palo Alto, CA) (25). Transfected cells were grown in RPMI 1640 medium, supplemented with L-glutamine, nonessential amino acids (BioSource International), 10% FBS (HyClone Laboratories, Logan, UT), and the antibiotics neomycin (Invitrogen, Carlsbad, CA), hygromycin B (Invitrogen), and/or puromycin (Clontech Laboratories).

Sources for each Ab are indicated: purified and PE-conjugated anti-human NKG2A mAb (Z199, mouse IgG2b), purified and PE-conjugated anti-human CD94 mAb (HP-3B1, mouse IgG2a), anti-human CD71 (YDJ1.2.2, mouse IgG1), F(ab')₂ goat anti-mouse-PE and isotype controls from Beckman Coulter (Fullerton, CA); PE-conjugated anti-mouse CD71 (C2, rat IgG1) and isotype control from BD Pharmingen (San Diego, CA); anti-SHP-1 rabbit polyclonal IgG and anti-phosphotyrosine mAb 4G10 (IgG2b) from Upstate Biotechnology (Lake Placid, NY). The NKG2A-specific mAb 8E4 (mouse IgG) was derived by Dr. J. P. Houchins (R&D Systems, Minneapolis, MN) (26). Transferrin Alexa 594 was purchased from Molecular Probes (Eugene, OR).

Flow cytometric analyses

Cells (0.1×10^6 in 200 μ l) were placed in 96-well flat-bottom plates in growing medium for 30 min at 4°C or 37°C. Saturating amounts of labeled or unlabeled anti-CD94 (HP-3B1) or anti-NKG2A (Z199) were added for different time periods. Cells incubated with labeled mAb were subsequently washed followed by immediate analysis with a FACSort cytometer (BD Immunocytometry, San Jose, CA). Cells incubated with unlabeled mAb were washed to eliminate unbound Ab, and then incubated with goat anti-mouse-PE for 30 min on ice followed by washing and analysis. In certain experiments, cells were treated with latrunculin A (LatA;

Calbiochem, San Diego, CA) or sodium azide (Sigma-Aldrich, St. Louis, MO) for 30 min before the addition of the mAb.

In some experiments for detection of cell surface stability and trafficking, cells were incubated with brefeldin A (BrefA; Calbiochem) and/or cyclohexamide (CHX; Calbiochem) for different time periods, and the cell surface expression of CD94/NKG2A, CD94/NKG2C/DAP12, or CD71 was monitored by FACS.

To investigate movement from intracellular compartments to the plasma membrane, all the CD94/NKG2A receptors expressed at the cell surface were blocked by incubating the cells with saturating amounts of unlabeled anti-NKG2A mAb (Z199) at 4°C for 30 min. After extensive washing, the cells were transferred to 37°C for different time periods to allow cycling between the plasma membrane and intracellular compartments. The cells were subsequently placed at 4°C, stained with anti-NKG2A-PE mAb (Z199), and analyzed by FACS.

Surface biotinylation, 2-mercaptoethanesulfonic acid (MESNA) treatment, immunoprecipitation, and immunoblotting

Internalization of cell surface receptors was measured as previously described (19). Briefly, cells (2×10^7 /ml) were washed three times with HBSS and incubated with 1 mg/ml of NHS-SS-biotin (Pierce, Rockford, IL) for 30 min on ice. Excess biotin was quenched by washing the cells three times with 25 mM lysine (Calbiochem). Cells were then cultured at 37°C for different time periods in the absence or presence of target cells (E:T is 1:1). Subsequently, cells were treated twice with 200 mM of MESNA (Sigma-Aldrich) for 10 min on ice. Cells were then washed two times with 5 mM iodoacetamide (Sigma-Aldrich) and lysed in lysis buffer (1% Triton X-100, 75 mM NaCl, 20 mM Tris (pH 8), 2 mM Na₃VO₄, 5 mM NaF, and 0.5 mM PMSF) and protease inhibitor mixture from Sigma-Aldrich. Immunoprecipitations were performed with anti-CD94 (HP-3B1) and anti-NKG2A (Z199) mAb and protein G (Invitrogen). Immunoprecipitates were washed three times in lysis buffer and boiled in nonreducing SDS buffer. Proteins were resolved in a 10% SDS-PAGE gel, transferred to Hybond-C Extra membranes (Amersham Pharmacia Biotech, Piscataway, NJ) and blots were probed with streptavidin-HRP (Amersham Pharmacia Biotech) and then developed using ECL Plus (Amersham Pharmacia Biotech). Membranes were stripped and reprobed with anti-NKG2A (8E4) mAb followed by donkey anti-mouse-HRP (Amersham Pharmacia Biotech). Densitometric analyses were conducted using Scion Image β 4.0.2 (Scion Corporation, Frederick, MD).

Confocal microscopic studies

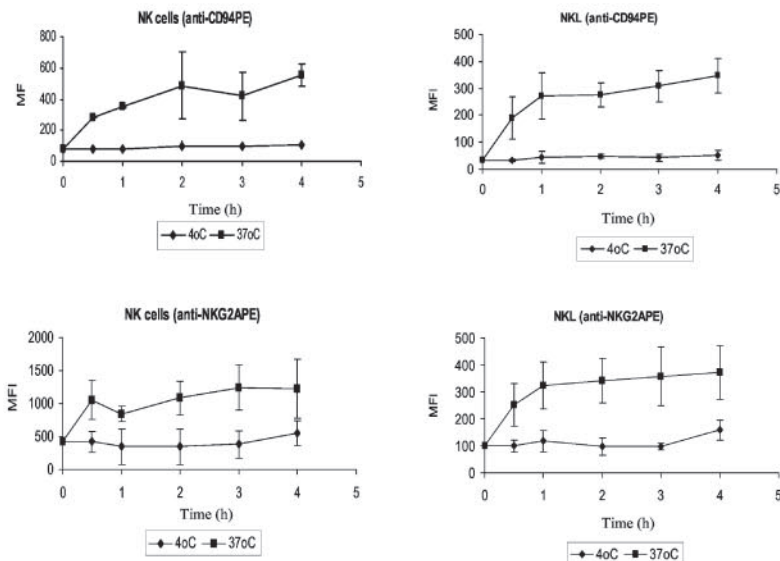
Anti-NKG2A mAb was labeled with Alexa fluor 488 or Alexa fluor 594 mAb labeling kits (Molecular Probes) according to the manufacturer's instructions. Cells (1×10^6 /ml) were incubated at 37°C or 4°C in growing medium for 1 h in the presence of mAb (5 μ g/ml) followed by washing with cold PBS and fixation with 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA). In some experiments, NKL cells were incubated with anti-NKG2A mAb labeled with Alexa 488 and transferrin Alexa 594. Images were collected on a Leica TCS-SP2 confocal microscope (Leica Microsystems, Exton, PA) equipped with argon (488 nm) and krypton (568 nm) lasers, using a $\times 63$ oil immersion objective NA 1.25. Detector slits were adjusted to minimize overlap between the channels. Images were processed with Imaris software v3.1.2 (Bitplane, Zurich, Switzerland) without filtering.

Results

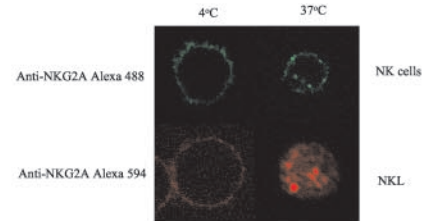
CD94/NKG2A receptors traffic to and from the cell surface

A constant level of expression of CD94/NKG2A receptors is maintained on the surface of CD94/NKG2A⁺ NK cells. This can be achieved by biosynthetic turnover (new synthesis and degradation), by stabilization of the receptor on the cell surface, and/or by recycling the receptors between the plasma membrane and cytoplasm. To determine whether CD94/NKG2A receptors cycle between the cell surface and intracellular compartments, we checked the uptake of labeled mAb (anti-CD94-PE or anti-NKG2A-PE) by CD94/NKG2A expressing polyclonal NK cells or the NKL cell line at 37°C for different time periods. The binding of conjugated mAb to CD94/NKG2A receptors increased linearly until it reached a plateau level after ~60 min as shown by an increase in the mean fluorescence intensity (MFI). No significant changes in binding capacity occurred when NKL cells or polyclonal NK cells were incubated at 4°C (Fig. 1A). Because the cell surface expression

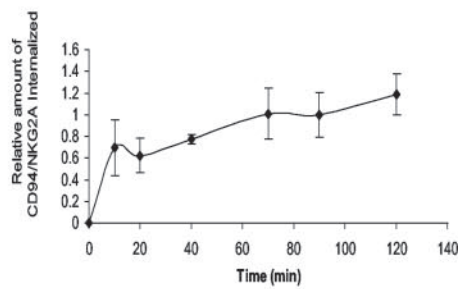
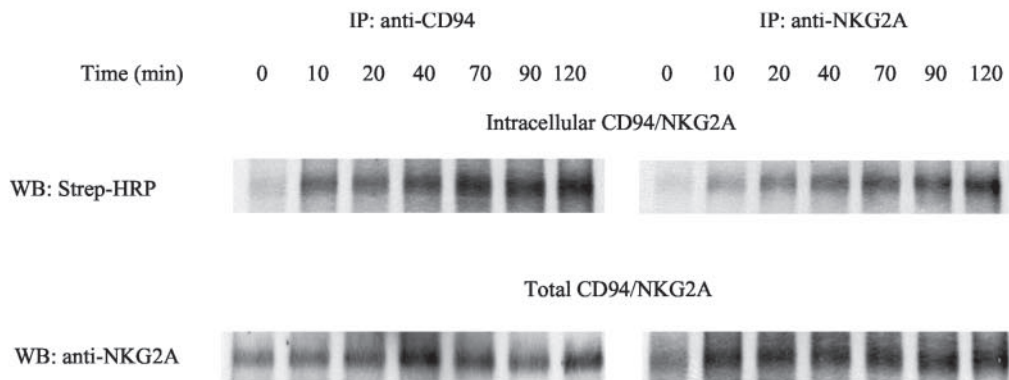
A



B



C



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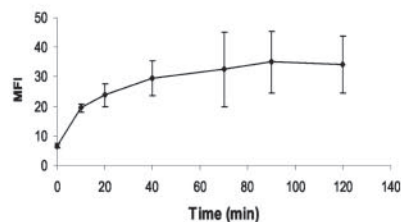


FIGURE 1. CD94/NKG2A continuously recycles between the cell surface and cellular interior. *A*, NKL or peripheral blood NK cells were incubated with PE-conjugated anti-CD94 mAb (HP-3B1) or anti-NKG2A mAb (Z199) at 37°C (■) or 4°C (◆) for different periods of time. Cells were then washed and analyzed by flow cytometry. MFIs were plotted vs time of incubation. *B*, NK cells (upper panel) or NKL cells (lower panel) were incubated with Alexa 488-conjugated or Alexa 594-conjugated anti-NKG2A mAb at 37°C or 4°C for 1 h, fixed, and examined by confocal microscopy. *C*, NKL cells were surface biotinylated with NHS-SS-biotin and incubated for various time periods at 37°C, followed by treatment with MESNA to (Figure legend continues)

level of CD94/NKG2A remained constant for the 4-h time period of the experiment (data not shown), the increase in the MFI of the cells incubated at 37°C with anti-NKG2A-PE mAb suggests that cell surface CD94/NKG2A receptors are internalized. The fact that the uptake of anti-CD94 mAb is severalfold higher than the uptake of anti-NKG2A mAb for both polyclonal NK cells and the NKL cell line probably reflects of the fact that both cell types express forms of CD94/NKG2 receptors other than CD94/NKG2A (data not shown). Similar results were obtained using Jurkat T cells transfected with CD94/NKG2A (data not shown). Confocal microscopic experiments were performed to directly prove that endocytosis of cell surface CD94/NKG2A receptors was occurring. Polyclonal NK cells were incubated for 1 h at 4°C or 37°C with anti-NKG2A mAb labeled with Alexa 488. Only cell surface labeling was detected when the cells were incubated at 4°C (Fig. 1B). In contrast, when cells were incubated at 37°C, CD94/NKG2A receptor-mAb complexes were not only detected on the cell surface, but also inside the cell. The same results were obtained with NKL cells.

To determine whether CD94/NKG2A receptors were internalized in the absence of bound mAb, NKL cells were treated with NHS-SS-biotin so that only surface CD94/NKG2A receptors were labeled. Cells were placed in culture at 37°C for increasing time periods, then treated with MESNA, a cell-impermeable reducing agent. This results in removal of the biotin label from CD94/NKG2A receptors remaining on the cell surface by reduction of the disulfide bond present in the labeling reagent. Because they are inaccessible to the cell-impermeable MESNA, internalized CD94/NKG2A receptors retain the biotin. After cell lysis, total CD94/NKG2A receptors were isolated by immunoprecipitation with anti-CD94 or anti-NKG2A mAb and the levels of biotinylated (intracellular) CD94/NKG2A receptors were determined by Western blotting with streptavidin-HRP. The results showed that the CD94/NKG2A receptors are rapidly internalized by NKL cells reaching a plateau at ~40 min (Fig. 1C). This rapid saturation of the intracellular compartments agrees with the results shown in Fig. 1A and they are similar to those obtained with the TCR using the same technique (19).

Next, we wanted to directly show that the intracellular pool of CD94/NKG2A receptors is continuously recycling between intracellular compartments and the plasma membrane. To do this, NKL cells were incubated with unconjugated anti-NKG2A mAb at 4°C to block cell surface expressed NKG2A. After washing, the cells were incubated at 37°C for different time periods to allow intracellular (unblocked) CD94/NKG2A time to cycle to the cell surface. The cells were then placed at 4°C again and stained with anti-NKG2A-PE mAb to specifically label only newly expressed CD94/NKG2A. The fact that a plateau is reached suggested that the receptor-mAb complexes do not dissociate intracellularly (Fig. 1D).

CD94/NKG2A receptors are long-lived and recycle through different cellular compartments than CD71 and CD94/NKG2C

Exocytosed CD94/NKG2A molecules (see Fig. 1D) could come either from the recycling pool of CD94/NKG2A, from newly synthesized CD94/NKG2A, or from a combination of both. To investigate these possibilities, NKL cells were preincubated with the

protein synthesis inhibitor CHX and then the recycling assay was performed in the presence of CHX. As seen in Fig. 2A, CHX did not change the rate at which CD94/NKG2A receptors arrived at the cell surface, verifying that the vast majority of newly expressed CD94/NKG2A molecules represents recycling rather than newly synthesized CD94/NKG2A.

BrefA causes tubulation and fusion of early endosomes with the *trans*-Golgi network and blocks anterograde transport from the endoplasmic reticulum to the Golgi complex (27, 28). The effect of BrefA on the expression of CD94/NKG2A and CD71 (transferrin receptor) by NKL cells was compared (Fig. 2B). Cell surface expression of CD71, which rapidly recycles (17), was reduced by BrefA treatment. In contrast, the level of CD94/NKG2A expression by NKL cells did not change significantly with treatment by BrefA during the 4-h time period examined. In light of the fact that CD94/NKG2A receptors are rapidly internalized and recycled back to the cell surface (Figs. 1 and 2A), the absence of effect by BrefA treatment suggested that internalized CD94/NKG2A recycles through compartments that differ from CD71. To confirm this, we analyzed the intracellular distribution of CD94/NKG2A compared with CD71. NKL cells were incubated at 37°C with anti-NKG2A labeled with Alexa 488 and transferrin labeled with Alexa 594 for different time periods and then analyzed by confocal microscopy. As it is shown in Fig. 2C, there is little or no colocalization of CD94/NKG2A with CD71, suggesting that these two receptors traffic through different compartments within the cell. As expected, the exposure of the cells to CHX for the same time period did not change the cell surface levels of CD94/NKG2A (data not shown).

NKG2C, an activating member of the NKG2 receptor family, associates with CD94 and DAP12 for expression on the cell surface (25). The extracellular regions of NKG2A and NKG2C, both of which recognize HLA-E as a ligand (8–10), are 92% identical (29). In contrast, while they are of similar length, their intracellular tails and transmembrane regions share only 60% identity. The intracellular tail of NKG2C also lacks the ITIMs present in NKG2A. Because they recognize the same ligand, comparison of their trafficking patterns was of interest. The mouse cell line Ba/F3 was transfected with CD94/NKG2C/DAP12 (25). In contrast to CD94/NKG2A, the cell surface levels of CD94/NKG2C/DAP12 decreased after BrefA treatment (Fig. 2D), strongly suggesting that these two receptors traffic through different compartments. Treatment with CHX did not significantly affect the cell surface expression of CD94/NKG2C/DAP12 (data not shown). This indicates that BrefA treatment affects an endocytic compartment and not endoplasmic reticulum:Golgi transport. These results also suggest that the trafficking of these NKG2 receptors is not controlled by CD94, as this chain is common to both the activating and inhibitory receptors.

Role of the ligand and ITIMs in CD94/NKG2A trafficking

The cell surface expression of HLA-E depends on the availability of signal sequence-derived peptides from certain HLA-A, B, and C, as well as HLA-G molecules (30). Thus, virtually all cells expressing classical HLA class I molecules also express HLA-E on the cell surface, including the NKL cell line, polyclonal NK cells,

remove extracellular biotin groups. After quenching with iodoacetamide, the cells were lysed in 1% Triton X-100. CD94/NKG2A was immunoprecipitated with anti-CD94 mAb (HP-3B1) or anti-NKG2A mAb (Z199), electrophoresed on SDS-PAGE, immunoblotted with streptavidin-HRP, and developed by chemiluminescence. Membranes were stripped and reprobed with an anti-NKG2A mAb (8E4), followed by goat anti-mouse-HRP. Densitometric analyses represent the mean of three independent experiments. The background at time 0 was subtracted, and the amount of internalized CD94/NKG2A was normalized to the total loaded amount of CD94/NKG2A as detected by immunoblotting. The data for anti-CD94 and anti-NKG2A were combined and plotted vs time. D, NKL cells were analyzed in the recycling assay as described in *Material and Methods*. MFIs were plotted vs time of incubation.

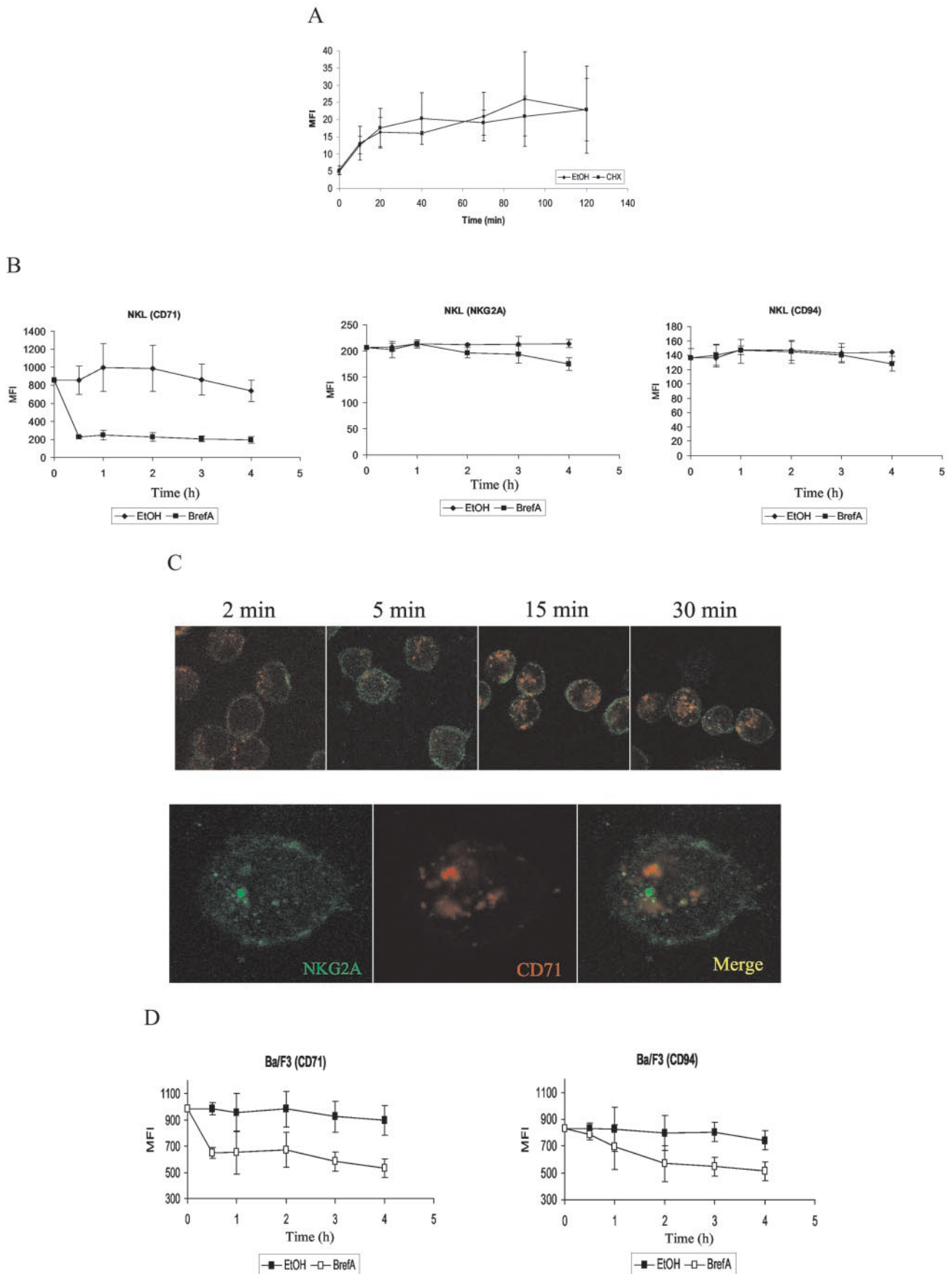


FIGURE 2. CD94/NKG2A and CD94/NKG2C/DAP12 are both long-lived receptors with different intracellular trafficking patterns. *A*, Untreated NKL cells and cells pretreated with CHX (50 $\mu\text{g/ml}$) were analyzed for constitutive exocytosis in the recycling assay as described in *Material and Methods*. MFIs were plotted vs time of incubation. All the experiments were performed three times. *B*, The effect of BrefA (10 $\mu\text{g/ml}$) on the cell surface expression of CD94, NKG2A, and CD71 expressed by NKL cells was analyzed by flow cytometry. *C*, Intracellular distribution of (Figure legend continues)

and CD94/NKG2A-transfected Jurkat T cells used in the previous experiments. Under the conditions used in these experiments, the cells were free to contact each other, which means that CD94/NKG2A molecules on the cell surface of one cell could be ligated by HLA-E expressed by adjacent cells. Thus, it is possible that the observed endocytosis and recycling of CD94/NKG2A receptors was induced by receptor-ligand interactions. Therefore, we examined the trafficking of CD94/NKG2A in the absence of the ligand. To do this, we used the HLA-E negative rat basophilic cell line RBL-2H3 that had been transfected with CD94 and NKG2A cDNAs. Analysis with conjugated mAb showed that CD94/NKG2A receptors expressed by RBL-2H3 cells are internalized similarly to those in the NKL cell line and polyclonal NK cells (Fig. 3A). Expression of NKG2A-EGFP fusion protein, along with CD94, in RBL-2H3 cells, showed NKG2A-EGFP localization in the plasma membrane and in intracellular vesicles. A movie recorded with living cells shows active contact between the plasma membrane and intracellular vesicular structures, suggesting that these vesicles serve as the means of intracellular transport (Fig. 3B and supplemental data).³ Furthermore, when the RBL-2H3 cells transfected with CD94/NKG2A-EGFP were incubated at 37°C with anti-NKG2A mAb conjugated with Alexa 594, intracellular colocalization of the conjugated mAb with CD94/NKG2A-EGFP was observed. This indicates that CD94/NKG2A receptor is transported to or within the cytoplasmic vesicles at least partially, if not solely, from the plasma membrane (Fig. 3C). To confirm that in RBL-2H3 cells CD94/NKG2A receptors are internalized at the same rate as NKL cells, we surface biotinylated RBL-2H3 cells and examine the rate of internalization after MESNA treatment (Fig. 3D). As can be seen, the rate of internalization is similar to NKL cells (see Fig. 1C). These results with RBL-2H3 cells verified that CD94/NKG2A cycles between the cell surface and cellular interior and that this trafficking is independent of ligation with HLA-E.

To further confirm that CD94/NKG2A trafficking occurs independent of interaction with its ligand, RBL-2H3 cells expressing CD94/NKG2A were mixed with target cells that do not (721.221) or do express HLA-E (721.221-Cw3) on the cell surface (9, 30). The interaction of CD94/NKG2A expressing RBL-2H3 cells with 721.221-Cw3 target cells induces the phosphorylation of CD94/NKG2A and association with SHP-1 (Fig. 3E). In contrast, no phosphorylation or SHP-1 coimmunoprecipitation occurred when the CD94/NKG2A expressing RBL-2H3 cells were mixed with the HLA-E-negative 721.221 target cells. These data are in agreement with previously reported results (11). Intracellular accumulation of cell surface biotinylated CD94/NKG2A receptors in RBL-2H3 cells showed very similar kinetics in the presence of either 721.221 target cells or 721.221-Cw3 HLA-E expressing target cells (Fig. 3F).

Tyrosines (Y) in intracellular motifs have been shown to play a role in endocytosis and trafficking of cell surface receptors. Proteins with the motif YXX ϕ (X, any amino acid; ϕ , a bulky hydrophobic amino acid) can bind the AP-2 complex, leading to association with clathrin-coated pits for internalization (16). Also, the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex are involved in the trafficking and down-regulation of TCR, suggesting a link between signaling and endocytosis (19, 31). As shown above (see Fig. 3), ITIMs in NKG2A are

phosphorylated upon receptor ligation, and these sites act as docking sites for the SHP-1 and SHP-2 phosphatases, thereby endowing inhibitory capacity (11, 12). These ITIMs (VIYSDL and ITYAEL) match the above-mentioned AP-2 binding motif. To investigate if the NKG2A ITIMs could function as AP-2-binding sites, we transfected RBL-2H3 cells with cDNAs for CD94 and a mutant form of NKG2A that encodes a molecule with Y to F substitutions in the ITIMs. The mutant CD94/NKG2A no longer functions as an inhibitory molecule as shown by its inability to recruit SHP-1 and to inhibit IgE-mediated serotonin release (12). Analysis of RBL-2H3 cells expressing this mutant CD94/NKG2A receptor for uptake of conjugated mAb, as well as examination for its internalization, showed that this receptor is internalized similarly to the wild-type receptor expressed in RBL-2H3 cells (Fig. 4). These data indicated that trafficking of CD94/NKG2A receptors does not depend on the YXX ϕ motif, and that the inability to transmit an inhibitory signal has no effect on receptor internalization.

CD94/NKG2A trafficking requires energy and an intact cytoskeleton

Treatment of cell lines with sodium azide has been shown to deplete ATP (32), thereby affecting cellular functions requiring this energy source. When NKL cells were cultured in the presence of sodium azide at 37°C, a decrease in the uptake of labeled mAb was detected in comparison with nontreated cells as measured by MFI. This decrease suggested that CD94/NKG2A trafficking to and from the cell surface is not a passive process, but requires energy (Fig. 5A).

LatA disrupts microfilament organization in cells by the formation of 1:1 complex with monomeric G-actin (33). LatA-treated NKL cells were placed in culture at 37°C with PE-conjugated anti-NKG2A mAb. A decreased uptake of labeled mAb was observed when the cells were incubated at 37°C compared with NKL cells cultured in the presence of solvent alone (Fig. 5B). These results suggest that the trafficking of CD94/NKG2A is dependent, at least in part, on the presence of an actin cytoskeleton.

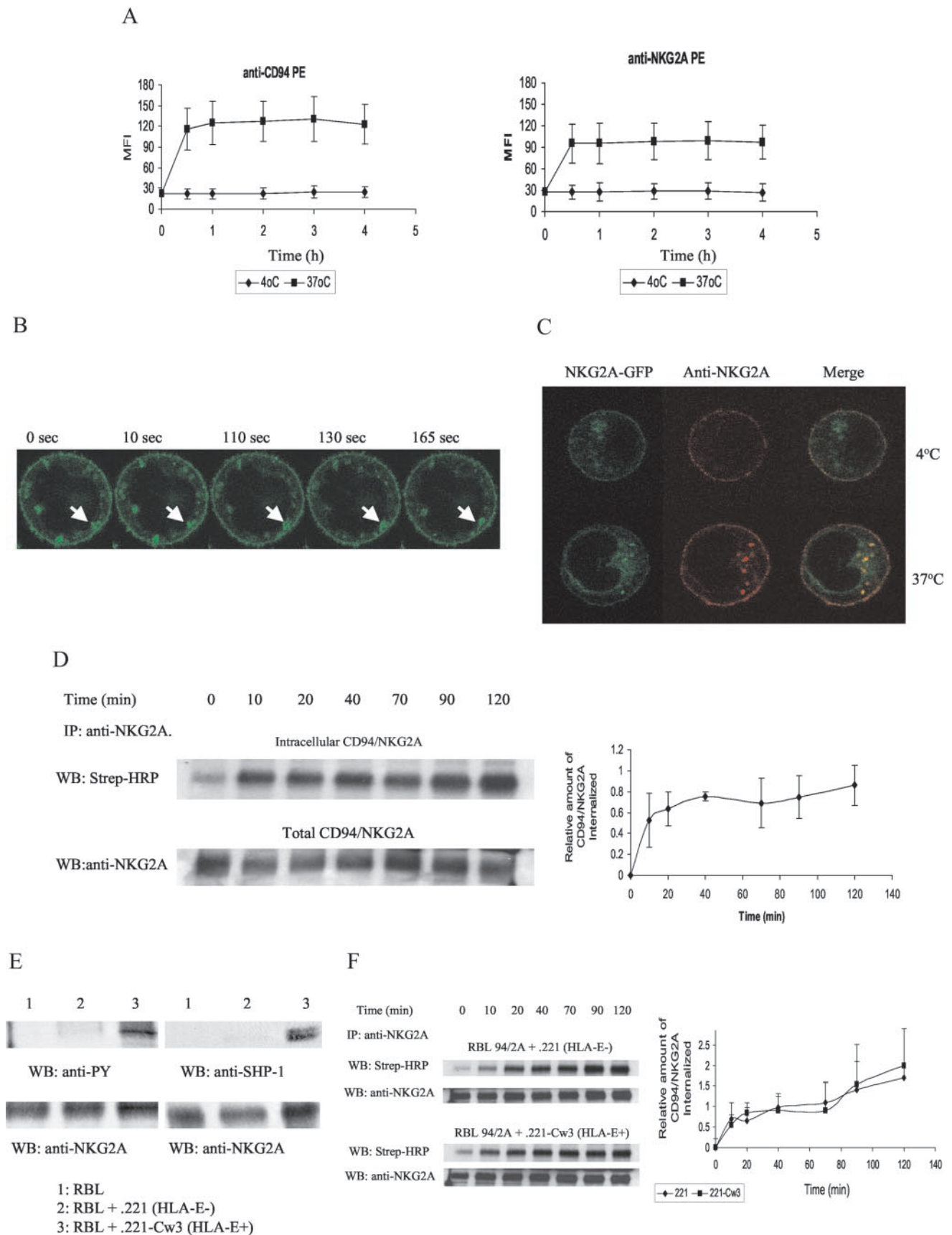
Discussion

Because NK cells are inherently poised to lyse all cells that they encounter, the maintenance of steady cell surface expression levels of inhibitory receptors that recognize ligands expressed on normal cells is essential to prevent self-destruction (15). In this study, we show that the human NK inhibitory receptor CD94/NKG2A continuously recycles between the cell surface and the cytoplasm, and that this is not influenced by ligation with HLA-E or transmission of the inhibitory signal resulting from this ligation. The recycling of CD94/NKG2A ensures a continuous supply of new receptors at the cell surface that are ready for ligand interaction and initiation of the inhibitory signaling cascade without the expenditure of excessive metabolic energy.

Selectivity in intracellular transport of membrane proteins is usually dictated by signaling motifs present in their cytoplasmic tails. Receptors containing the YXX ϕ motif often are clustered to characteristic invaginations in the plasma membrane whose cytoplasmic surfaces are coated with clathrin for internalization (16). CD94/NKG2A receptors contain in their ITIMs the sequence YXXL that fits the YXX ϕ motif. However, we showed that the substitution of Phe for Tyr residues in the ITIMs had no impact on

³ The on-line version of this article contains supplemental material.

CD94/NKG2A and CD71 in NKL cells. NKL cells were allowed to internalize anti-NKG2A Alexa 488 (green) and transferrin Alexa 594 (red) and then were analyzed by confocal microscopy. *Upper panel*, The internalization at different periods of time; *lower panel*, a representative cell at a higher magnification. *D*, The effect of Bref A (10 μ g/ml) on the cell surface expression of CD71 and CD94 expressed by Ba/F3 cells transfected with CD94/NKG2C/DAP12 was analyzed by flow cytometry. All the experiments were performed at least three times.



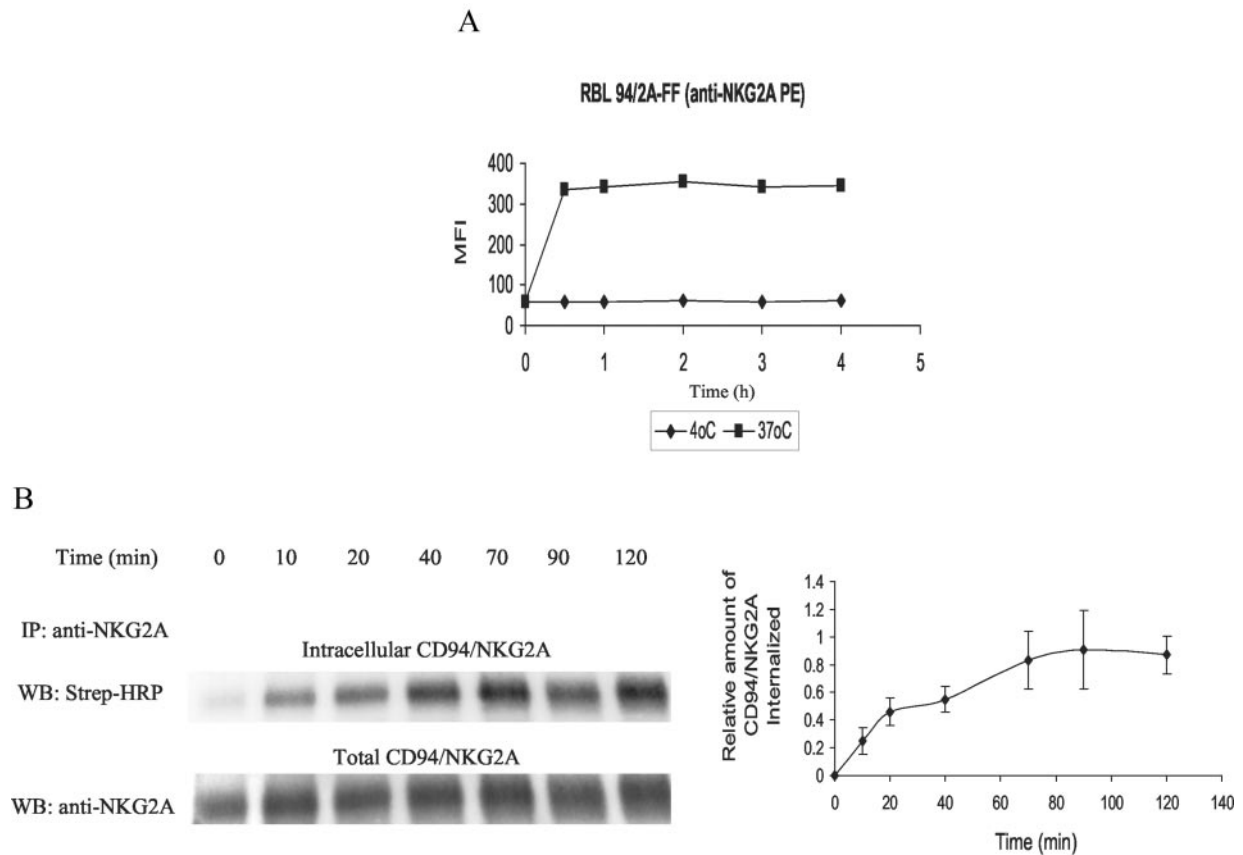


FIGURE 4. CD94/NKG2A is internalized and recycles in the absence of inhibitory signals. RBL-2H3 cells transfected with CD94/NKG2A containing Tyr for Phe substitutions in its ITIMs were used in this experiment. *A*, Uptake of PE-conjugated anti-NKG2A mAb was monitored as described in Fig. 1*A* at 4°C and 37°C. *B*, internalization was examined as described in Fig. 1*C*.

the CD94/NKG2A trafficking pattern, thereby ruling out that the ITIMs within CD94/NKG2A function as YXX ϕ motifs. This agrees with results reported by Minskoff et al. (34) that showed that Fc γ RIIB trafficking is not regulated by ITIM motifs. In support of this finding, we have also shown that Con A pretreatment of NKL cells failed to interfere with the uptake of anti-NKG2A mAb, while the uptake of anti-CD71 mAb is clearly affected (our unpublished observations). Con A is an agent that blocks clathrin-dependent endocytosis (35). These observations suggest that CD94/NKG2A internalization is clathrin-independent. This is in contrast to TCR recycling, which depends at least in part on the YXX ϕ motif that is present in the ITAMs of the TCR-CD3 complex (19, 36).

TCR-CD3 complexes continuously recycle between the cell surface and the cytoplasm in resting T cells. Ligation by MHC:peptide complexes leads to rapid down-modulation from the cell surface (21). This down-modulation is due to intracellular retention and degradation of ligated TCR-CD3 (19). Recent evidence suggests that ligand-induced TCR down-regulation and constitutive TCR recycling may be independent processes (20). In any case, it is clear that T cells can differentiate between ligated and unligated TCR-CD3, directing each type of complex to different locations

within the cell. CD94/NKG2A receptors on peripheral blood NK cells as well as on almost all human cell lines such as NKL and CD94/NKG2A-transfected Jurkat T cells continuously interact with the HLA-E ligand. Thus, it can be assumed that under physiological conditions there is continuous ligation of CD94/NKG2A by HLA-E expressed on autologous cells in close proximity. Our data show that CD94/NKG2A is a very stable protein with a slow degradation/synthesis rate, which suggests that NK cells do not degrade ligated CD94/NKG2A receptors, but rather reuse them again and again. It remains unknown if NK cells can differentiate between ligated and unligated receptors and if they traffic through different compartments. Our data with RBL-2H3 cells, which do not express HLA-E, suggest that ligated and unligated CD94/NKG2A receptors recycle in a similar pattern. The results obtained by exposing the RBL-2H3 cells to HLA-E-negative and HLA-E-positive target cells further supports this conclusion (Fig. 3*F*).

The cell surface expression of HLA-E is low on most human cells (24). With this in mind, it is not known if these levels are sufficient to sustain NK cell inhibition by ligating a few CD94/NKG2A receptors on NK cells or if HLA-E molecules on target cells can serially trigger CD94/NKG2A to maintain the inhibitory signal. If serial triggering plays a role it could be facilitated by the

by confocal microscopy. Colocalization is shown by the yellow color. *D*, CD94/NKG2A internalization by transfected RBL-2H3 cells was determined as described in Fig. 1*C*. *E*, RBL-2H3 cells transfected with CD94/NKG2A were incubated with 721.221 or 721.221-Cw3 cells at E:T ratio of 1:2 for 3 min at 37°C. Cells were then lysed and CD94/NKG2A was immunoprecipitated with anti-NKG2A mAb (Z199). After SDS-PAGE, blots were probed with anti-phosphotyrosine mAb (4G10) or with anti-SHP-1 mAb and developed with secondary Ab-HRP. Blots were stripped and reprobed with anti-NKG2A mAb (8E4) to determine the total loaded amount of CD94/NKG2A. *F*, CD94/NKG2A internalization by transfected RBL-2H3 cells was determined as described in Fig. 1*C*, except that RBL-2H3 cells were incubated in the presence of 721.221 cells or 721.221-Cw3 cells (E:T ratio is 1:1).

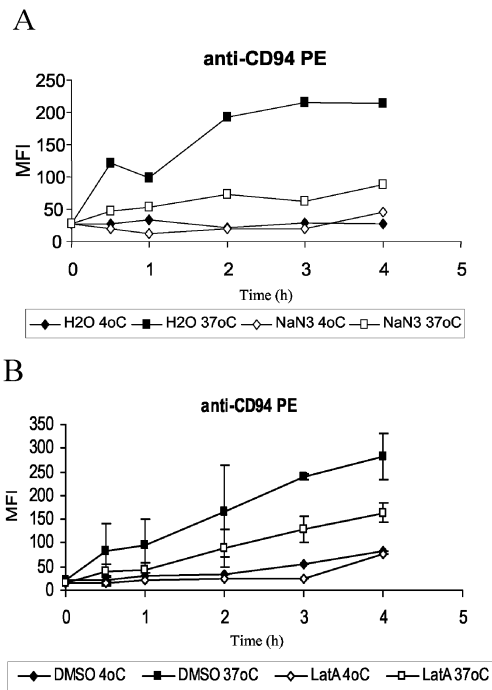


FIGURE 5. CD94/NKG2A trafficking can be inhibited by depletion of cellular ATP and by impairing cytoskeleton polymerization. *A*, NK cells were pretreated with 80 mM sodium azide for 30 min. PE-conjugated anti-CD94 mAb was added and cells were incubated at 37°C and 4°C. Cells were then washed and analyzed by flow cytometry as in Fig. 1. Similar results were obtained with RBL-2H3 cells transfected with CD94/NKG2A (data not shown). *B*, As described for *A*, except cells were pretreated with 20 nM LatA.

very fast association and dissociation rate constants that characterize the interaction between HLA-E and CD94/NKG2A (37). The potential for sequential engagement of multiple receptors by a few ligands is reminiscent of the serial triggering of TCR by a small number of MHC:peptide complexes (21). However, in contrast to the down-regulation observed after TCR triggering, the ligation of CD94/NKG2A on NK cells does not result in receptor down-modulation. It is possible that the number of receptors down-modulated by ligation is relatively small in comparison to the total number of receptors undergoing recycling and thus they are not readily distinguishable. Whatever the case, our findings are in agreement with previous data showing that the incubation of CD94/NKG2A⁺ NK cells with heterologous target cells expressing the HLA-E ligand does not result in down-regulation of cell surface expression of CD94/NKG2A (38).

Despite the fact that they both recognize HLA-E, our results using BrefA as an inhibitor showed that CD94/NKG2A traffics through different compartments than CD94/NKG2C/DAP12 (see Fig. 2). We are confident that this difference is not due to the expression in different cell types, but more likely to differences in signaling motifs in the cytoplasmic domains of these receptors. NKG2C possesses a dileucine signaling motif that is commonly used for endocytic processing (16), whereas CD94 and NKG2A lack dileucine motifs in their cytoplasmic tails. Examples of other molecules containing this motif are CD3 γ and δ (20, 31). The dileucine motif within NKG2C has the sequence DxxxLL, which is identical with the sequence required for the internalization of CD3 γ (20, 31). We are in the process of determining if the dileucine motif accounts for the distinctive trafficking patterns of CD94/NKG2A and CD94/NKG2C/DAP12. The association of

CD94/NKG2C with DAP12 could also explain the different trafficking patterns of these two NKG2 family receptors. DAP12 contains a Yxx ϕ motif that is coincident with its ITAM sequence. As mentioned previously, the ITAMs in the TCR-CD3 complex are known to play a role in TCR trafficking (19, 36).

Although the treatment of cells with BrefA interferes with the expression levels of many cell surface proteins, the failure to affect cell surface expression of CD94/NKG2A is not unique to this receptor. For example, when internalized, the insulin-sensitive glucose transport protein GLUT4 is targeted to a yet to be identified intracellular structure, and this processing step is resistant to BrefA (39). Moreover, the insulin-dependent recruitment of GLUT4 to the cell surface is BrefA-resistant as well (40). GLUT4 contains the motif Phe-Gln-Gln-Ile that is responsible for the transport of internalized GLUT4 to a specific intracellular compartment. NKG2A does not have this motif, suggesting that CD94/NKG2A may traffic, at least in part, through cellular compartments not shared with GLUT4 even though they share resistance to the action of BrefA.

Based on the results obtained with sodium azide and LatA, we have shown that internalization and trafficking of CD94/NKG2A is an active cellular process that requires cytoskeletal rearrangement. The trafficking of other receptors, such as CD71, is also dependent on actin polymerization (41), and TCR-CD3 trafficking has been shown to be sensitive to latrunculin compounds (42). It is likely that LatA exerts its effect on CD94/NKG2A trafficking by interfering with translocation from the plasma membrane to intracellular compartments in a fashion similar to the way latrunculin B blocks the targeting of BCR from the plasma membrane to late endosomes and lysosomes (43).

Relatively rapid internalization and recycling of CD94/NKG2A receptors is likely a process that has evolved to maintain stable functional expression while NK cells are in continuous contact with ligand expressing target cells. Under normal conditions, most CD94/NKG2A receptors should rapidly encounter HLA-E ligand on neighboring cells. This likely induces phosphorylation and SHP-1 association (see Fig. 3), thereby initiating inhibitory signals. Cross-linking of CD94/NKG2A on transfected RBL-2H3 cells showed colocalization of the receptor with SHP-1 in the membrane and inside the cell (12). This suggests that these complexes travel from the membrane where the receptors are ligated to the cellular interior. CD94/NKG2A phosphorylated at the cell surface due to ligand engagement may have to cycle through a specific compartment to be dephosphorylated before recycling back to the cell surface to begin the process again. Although it is possible that the transmission of this inhibitory signal, as well as receptor “reconditioning”, may require receptor internalization, it is clear from our studies with transfected RBL-2H3 cells and 721.221 cells (see Figs. 3 and 4) that initiation of the inhibitory signal is not a requisite for receptor recycling.

In conclusion, the ligation of CD94/NKG2A by HLA-E on target cells initiates an inhibitory signal that stops activation processes by NK cells that would otherwise lead to unrestrained bystander killing (autoimmunity). Thus, the maintenance of constant and optimal levels of CD94/NKG2A on the cell surface is likely a requirement for regulating inappropriate NK cell activity. This consistent availability of CD94/NKG2A receptors at the cell surface is achieved by the reuse of the receptors in a relatively fast recycling process, without any apparent ligand-induced degradation of the receptors. The fast on and off dissociation rates that characterize the CD94/NKG2A:HLA-E interaction may serve to facilitate this process (37).

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