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FRONTLINES

Direct Binding of Purified HLA Class I Antigens by Soluble NKG2/CD94 C-Type Lectins from Natural Killer Cells

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Human natural killer (NK) cell cytotoxicity is inhibited following human leucocyte antigen (HLA) class I binding by killer cell inhibitory receptors belonging to the immunoglobulin or C-type lectin protein families. Of the latter family, CD94 and NKG2A or -B associate to inhibit NK cell cytotoxicity. We have constructed C-Myc epitope-tagged soluble NKG2A, -B, -C, -D and human NKR-P1 lectin domains, and studied their ability to associate with Flu-tagged soluble CD94 lectin domains. Furthermore, their ability to bind solubilized immunoaffinity-purified HLA class I antigens, either alone or following association with CD94 lectin domains, was evaluated using flow cytometry and Western blot analyses. We show that soluble NKG2A, -B and -C lectin domains interact with CD94 lectin domains to form complexes, whereas NKG2D and human NKR-P1 lectin domains do not. Soluble NKG2C, -D and CD94 lectin domains bind solubilized affinity purified HLA class I antigens on their own, whereas NKG2A and -B require association with CD94 lectin domains for binding. Soluble human NKR-P1 lectin domains do not bind solubilized HLA class I antigens in our system.

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INTRODUCTION

Virus-infected cells and tumour cells that have escaped T-cell-mediated immunity by down-regulating major histocompatibility complex (MHC) class I expression can be killed by natural killer (NK) cells. Kärre and coworkers found that the sensitivity to NK-mediated killing was inversely correlated to the surface density of MHC class I molecules on target cells, i.e. the lower the MHC class I surface expression the higher the sensitivity to NK-mediated lysis [1]. This observation was explained at the molecular level when a family of type II proteins with C-type lectin domains [2, 3] and a family of immunoglobulin (Ig)-homologous proteins with type I membrane topology [4–6] were identified on murine and human NK cells, respectively, and shown to inhibit NK cell cytotoxicity upon binding to human leucocyte antigen (HLA) class I molecules. These receptors were termed as killer cell inhibitory receptors (KIRs). KIRs of the Ig superfamily are clonally distributed and specific for HLA-A, -B, and -C supertypic epitopes [7, 8]. Their inhibitory effect is brought about by a protein tyrosine phosphatase, SHP-1, which is recruited to the receptor by binding to Src family kinase-phosphorylated cytoplasmic tyrosine-containing motifs [9–11]. Stimulatory receptors belonging to this family bind HLA class I antigens but lack cytoplasmic immune-receptor tyrosine-based inhibitory motifs (ITIMs) [7, 8, 12]. Mouse KIRs belonging to the Ig superfamily are yet to be identified. In mice, however, members of the C-type lectin superfamily, i.e. Ly49, have been shown to inhibit NK cytotoxicity upon binding to mouse H-2 molecules [13, 14]; Ly49A was shown to bind H-2D^d and H-2D^k – an interaction that was at least partly carbohydrate-dependent [15]. Even though Ig domain-homologous KIRs and Ly49 belong to different protein families, they both contain cytoplasmic ITIMs explaining their similar functionality. Rat NKR-P1, also a member of the C-type lectin family, was suggested to be an activation receptor [16, 17] and has been implied to bind carbohydrates on NK-sensitive tumour cells [18, 19]. In humans, NKG2A-E [20], CD94 [21] and human NKR-P1 [22] are members of the C-type lectin family of NK receptors, genes of which map in the NK gene complex on the short arm of human chromosome 12.

Moretta and coworkers have suggested that CD94 is a specific receptor for HLA-B alleles of the Bw6 serotype, based on the ability of certain NK cell clones to kill Epstein–Barr virus-transformed B

* The contribution of the first two authors should be considered equal.

lymphoblastoid cell lines expressing HLA-B7 in the presence, but not in the absence, of CD94 antibodies [23]. However, others have observed CD94-expressing NK cell clones that fail to recognize HLA-B7, and instances where the effect of anti-CD94 antibodies on HLA recognition was found to be more complex [21, 24, 25]. Nonclassical HLA class I molecules like HLA-G [26] and, more recently, HLA-E [27, 28] have also been shown to bind CD94/NKG2 inhibitory molecules. In the latter case, HLA-E binding was dependent on its ability to present classical HLA class I allelic leader peptides [27, 28]. Based on reversed ADCC assays, CD94 antibodies have been shown to either augment, inhibit, or have no effect on NK cell-mediated cytotoxicity and cytokine production [24]. Studies initiated based on the fact that the CD94 cytoplasmic tail is very short and may not be capable of transducing signals upon ligand binding, revealed that CD94 was covalently associated with a tyrosine-phosphorylated protein [29], which was later shown to be identical to NKG2A [30, 31]. The CD94/NKG2A complex was inferred to be an NK-inhibitory receptor complex based on the presence of ITIMs in NKG2A cytoplasmic tails. Indeed, chimeric receptors carrying NKG2A cytoplasmic tails have been shown to inhibit NK cell cytolytic activity following crosslinking, whereas chimeric proteins with the NKG2C cytoplasmic tail stimulated NK calcium mobilization and lytic activity [32]. Association of NKG2A, -B, -C and -E with CD94 on the cell surface of transfected 293T cells was inferred from flow cytometry using a rabbit anti-CD94-associated protein (anti-CD94AP) serum depleted of anti-CD94 antibodies [33]. $CD94^+$ 293T cells transfected with the NKG2D cDNA gave minimal reactivity with the antiserum. Whether this was due to a lack of reactivity of the CD94AP antiserum with the NKG2D/CD94 complex, or due to an inability of NKG2D to associate with CD94, was not clear from this study [33]. No direct binding of putative NKG2A-E/CD94 complexes to HLA class I molecules has been established.

We have constructed C-Myc epitope-tagged soluble NKG2A, -B, -C, -D and hNKR-P1 lectin domains, and studied their ability to associate with Flu-tagged soluble CD94 lectin domains. Furthermore, their ability to bind solubilized purified HLA class I antigens individually or following association with CD94 lectin domains was evaluated using flow cytometry and Western blot analyses.

MATERIALS AND METHODS

Cell culture. COS-7 m6 cells were passaged in Dulbecco's modified Eagle's medium (DMEM), with 10% fetal bovine serum (FBS) and 25 μg/ml gentamicin sulphate. Mouse hybridomas Myc 1–9E10.2 (No. CRL-1729) secreting anti-Myc tag antibodies and W6/32 (no. HB-95) secreting anti-HLA class I antibodies were obtained from ATCC (Rockville, MD, USA), and cultured in HEPES-buffered RPMI 1640 with 10% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Monoclonal antibodies. Mouse anti-Myc tag (9E10) and anti-HLA class I (W6/32) antibodies were purified from hybridoma supernatants on goat antimouse IgG agarose beads (A-6531, Sigma, St Louis, MO, USA). Briefly, hybridoma supernatants were passed through a 9-mm in diameter column containing 5 ml packed antimouse IgG agarose beads, washed with 100 ml each of 10 mM Tris-HCl pH 7.5 with 300 mM NaCl and 0.1% Triton X-100, and phosphate-buffered saline (PBS), and then eluted with 0.1 M Tris-glycine pH 2.5 with 150 mM NaCl. Eluted proteins were immediately buffered, dialysed against PBS and lyophilized. Purified W6/32 MoAbs were biotin-labelled using a biotinylation kit (cat. no. 141 8165) according to the instructions of the manufacturer (Boehringer-Mannheim, Mannheim, Germany). The mouse monoclonal anti-Flu epitope tag antibody specific for a peptide derived from the influenza haemagglutinin (see below) was purchased from Boehringer-Mannheim (cat. no. 158 3816) and peroxidase-conjugated affinity-purified polyclonal goat antimouse IgG_{Fc} specific antibodies (cat. no. 555 66) was from Cappel/Organon Teknika (Durham, NC, USA).

Construction of expression vectors. Full-length NKG2A, NKG2B and NKG2C were polymerase chain reaction (PCR) amplified from a NK cell cDNA library in CDM8 (C. Romeo and B. Seed, unpublished observations) using sense primers containing 6 codons of complementarity to the 5′ end of the coding sequence, a consensus translational start sequence, and an *Xho*I restriction site, together with antisense primers containing 6 codons of complementarity to the 3′ end of the coding sequence, a consensus translational stop, and a *Not*I restriction site. Full-length NKG2D, CD94 and human NKR-P1 were PCR-amplified in the same manner except that we used *Hin*dIII as the restriction site in the upstream sense primers. Full-length PCR products were cloned into the polylinker of the eukaryotic expression vector, CDM8, using *Xho*I and *Not*I or *Hin*dIII and *Not*I, respectively [34]. The correct sequence of each C-type lectin cDNA clone was verified by the dideoxy chain termination method using modified T7 DNA polymerase (Sequenase™, Amersham, Sweden AB, Solna, Sweden).

Expression vectors encoding epitope-tagged, secreted, and soluble NK cell C-type lectin domains were constructed by PCR-amplifying their carboxy termini downstream of the transmembrane domains and subcloning these fragments with preserved reading frame into *Xba*I and *Not*I restriction sites of CDM7-based vectors carrying the cDNA sequences encoding the Myc (EQKLISEEDLN)- or Flu (YPYDVPDYAS)-epitope tags preceded by the CD5 signal peptide sequence upstream of the cloning site (J. Haas and B. Seed, unpublished observations). The same *Not*I 3′ primers as above were used together with forward primers having the following sequences:

NKG2A: 5′-cgc ggg tct aga aca gag gca caa caa ttc-3′;
NKG2B: 5′-cgc ggg tct aga acg tca ttg tgg cca ttg-3′;
NKG2C: 5′-cgc ggg tct aga gga gca gaa caa ttc ttc-3′;
NKG2D: 5′-cgc ggg tct aga caa cca aga agt tca aat-3′;
hNKR-P1: 5′-cgc ggg tct aga gaa atc atc aat aga aaa-3′;
CD94: 5′-cgc ggg tct aga ttc ttt tac taa act gag-3′.

PCR conditions were such as to keep the mutation rates low, i.e. large amounts of template plasmid and few cycles were used.

Production and purification of secreted epitope-tagged NK cell C-type lectin domains. COS m6 cell were transfected using the diethyl amino ethyl (DEAE)-dextran protocol and 1 μg of CsCl gradient-purified plasmid DNA per ml transfection cocktail [35]. COS cells were transfected at approximately 70% confluency with empty vector (CD5lMycEγ1 or CDM8), individual Myc-tagged, soluble NKG2A-D, hNKR-P1 or CD94 lectin domain-encoding expression plasmids, or with plasmids encoding Myc-tagged NKG2A-D or hNKR-P1 lectin domains in combination with the plasmid encoding the Flu-tagged CD94 lectin domain. Transfected cells were trypsinized and transferred to new flasks the day after transfection [35]. Following adherence for approximately 12 h, the medium was discarded, cells washed with PBS, and subsequently incubated another 7 days in serum-free, AIM-V medium

(cat. nr. 12030, Life Technologies Inc., Grand Island, NY, USA) [35]. After incubation, supernatants were collected, debris spun down (1400×***g***, 20 min), and NaN_3 added to 0.02% [35]. Myc-tagged NK cell lectin domains from 8 ml of supernatant were purified on 100 μl CNBr-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) conjugated with mouse anti-Myc tag antibodies (see above) by rolling head over tail, overnight at 4°C. The beads were collected by centrifugation, washed three times in PBS, and subsequently used for SDS-PAGE and Western blot analysis.

SDS-PAGE and Western blotting. SDS-PAGE was run by the method of Leammli with a 5% stacking gel and a 10 or 15% resolving gel using a vertical Mini-PROTEAN II electrophoresis system (Bio-Rad, Herculus, CA, USA) [36]. Separated proteins were electrophoretically blotted onto Hybond™-C extra membranes (Amersham) using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) [37]. Protein gels were stained using a silver staining kit according to the manufacturer's instructions (Bio-Rad). Following blocking for at least 2 h in 3% BSA in Tween 20 in Tris-buffered saline (TTBS: 10 mM Tris-HCl pH 8.0 with 150 mM NaCl and 0.05% Tween 20), the membranes were probed for 30 min at room temperature with anti-Myc or anti-Flu tag antibodies (5 μg/ml in 3% BSA/TTBS) to detect Myc-or Flu-tagged soluble C-type NK cell lectins, washed three times in PBS, and then again incubated for 30 min in room temperature with a peroxidase-conjugated goat antimouse IgG antibody (5 μg/ml in 3% BSA/TTBS). After washing three times in PBS, bound secondary antibody was visualized by chemiluminescence using the ECL™ kit according to the instructions of the manufacturer (Amersham). To detect HLA class I antigens bound to paramagnetic beads carrying the lectin domains (see below), 20 μl of the magnetic beads were mixed with an equal volume of 2×reducing sample buffer, boiled and analysed on a 15% SDS-PAGE. Following blotting to Hybond™-C extra membranes, HLA class I antigens were detected with a biotinylated anti-HLA-A, -B, and -C antibody (see above) at 7 μg/ml in 3% BSA/TTBS, peroxidase-conjugated avidin (cat.no. 55898; Cappel/Organon Teknika), and chemiluminescence (ECL™ kit; Amersham).

Isolation of HLA class I antigens. HLA class I molecules were affinity-purified from solubilized platelets obtained from human platelet-rich plasma from 120 individuals using the method of Parham with slight modifications [38, 39]. Protein purity was checked by SDS-PAGE and Western blot analysis. Using allele-specific antisera, the HLA class I antigen pool was shown to contain 24 different HLA-A allotypes, 35 different HLA-B and 6 different HLA-C allotypes. Of the more common allotypes, HLA-A9, -A19, -A23, and -B14 were not present in the pool.

Flow cytometry. One hundred μl of specific immunoadsorbents were prepared using commercially available goat antimouse Ig Fc-specific-coated paramagnetic beads (BioMag, cat. no. 8-43440, Perseptive Biosystems, Framingham, MA, USA). Mouse anti-Myc tag antibodies (9E10) at a concentration of 1 mg/ml were bound to goat antimouse Fc-specific antibody-coated magnetic beads by rolling for 60 min at 4°C. A preclearing step was used to reduce nonspecific binding of COS cell supernatant-derived proteins other than the soluble Myc-tagged NK cell C-type lectin domains. For this purpose, 1 ml of the supernatants was mixed by rolling, overnight, at 4°C with 100 μl of goat antimouse antibody-coated paramagnetic beads. The beads were removed using a magnetic stand housing the tube. In addition, 2 ml of supernatants were treated in the same manner. The beads were again removed using a magnet, and a total of 3 ml precleared supernant was used to absorb the soluble domains on anti-Myc tag-coated magnetic beads. An additional preclearing step was performed to reduce nonspecific binding of anti-HLA class I (W6/32) phycoerythrin-conjugated antibodies to anti-Myc tag-coated beads. For this purpose, 100 μl of PE-conjugated anti-HLA class I antibodies (1 mg/ml; clone W6/32, cat. no. R7000, Dako A/S, Glostrup, Denmark) were preabsorbed with 100 μl of anti-Myc tag-coated magnetic beads by rolling at 4°C overnight. The beads were removed using a magnet. The preabsorbed PE-conjugated anti-HLA class I antibodies were later used for detection of bound purified HLA class I antigens to NK cell C-type lectin domains.

Thirteen sets of beads were prepared, one for each soluble domain alone (six sets), one each for the Flu-tagged sCD94 encoding vector cotransfected with the others (five sets), and two sets for the negative controls (CDM8 and CD5lMycEγ1 + Flu-tagged sCD94, respectively). The beads were washed three times with PBS by isolating the beads after each wash with a magnet.

One hundred μl of each set of anti-Myc-tag antibody-coated magnetic beads were incubated with 1 ml of the respective precleared supernatants for 4 h at 4°C. The beads were isolated with the magnet, another 1 ml of supernatant was added to the respective beads, and the procedure was repeated. In total this procedure was repeated three times, with each incubation done at 4°C for 4 h and with 1 ml of precleared supernatant. After the third incubation, each set of beads was washed three times with PBS. Washed beads were resuspended in 100 μl of the same buffer.

Fifty μl of each set of NK cell C-type soluble domain-coated magnetic beads were incubated with 50 μl of purified HLA class I antigens (0.833 mg/ml) overnight at 4°C on a rock 'n roller. The beads were isolated using a magnet and washed three times using PBS. Five μl of 1 : 4 diluted PE-conjugated anti-HLA class I antibodies were added and incubated in the dark at 4°C for 20 min. The beads were washed twice with PBS and resuspended in 500 μl of PBS containing 0.5% BSA. The beads were then ready to be analysed in the flow cytometer.

A fluorescence-activated cell analyser (FACSsorter, Becton Dickinson, Mountain View, CA, USA) with an argon laser producing 400 mW of light at 488 nm was used for all analyses. Data were collected with logarithmic amplification and fluorescence intensity was displayed on a 256-channel, 4-decade log scale delineated in arbitrary log units. Fluorescence signals from 10 000 beads were counted and the percentage of PE-positive beads was recorded. Histograms of number of beads vs. log fluoresence intensity were generated.

RESULTS AND DISCUSSION

The extracellular parts of human NK-cell C-type lectins, NKG2A-D, CD94 and hNKR-P1, can be expressed as soluble proteins

In order to investigate the ability of human NK cell C-type lectins to associate with each other and to bind to purified solubilized HLA class I antigens, we created Myc- and Flu-epitope tagged soluble NK lectin receptors. The human NK cell C-type lectin family has been shown to be expressed as disulphide-linked dimers [22, 33, 40], and for rat NKR-P1 cysteines in the juxtamembrane stalk region as well as in the C-terminal carbohydrate recognition domain were important for efficient dimerization [18]. Therefore, we made soluble NK lectins that included the stalk region by using sense primers starting just 3′ of the transmembrane domain. Expression of Myc-tagged soluble NK cell lectins in the supernatants of transfected COS cells was verified with SDS-PAGE and Western blotting using an anti-Myc tag antibody (Fig. 1). Following transfection of vectors encoding soluble NKG2A, -B, -C, -D, hNKR-P1 and CD94, the anti-Myc tag antibody detected protein products in the crude

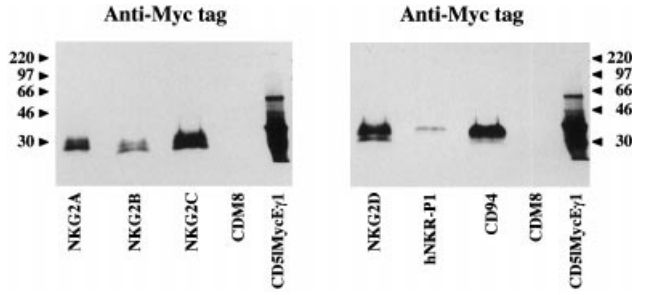

Fig. 1. Western blot analysis of soluble natural killer (NK) cell C-type lectins using an anti-Myc tag antibody to identify secreted Myc epitope-tagged proteins in supernatants of transfected COS cells. Fifteen μl of crude supernatant from COS cells transfected with expression vectors encoding the extracellular parts of NKG2A, -B, -C, -D, hNKR-P1 and CD94 were boiled together with 15 μl of reducing sample buffer, and separated on a 10% SDS-polyacrylamide gel. The CDM8 vector was used as negative control and a Myc-tagged human IgG Fc protein encoded by the CD5lMycEγ1 vector was used as a positive control. The molecular weight and migration length in the gels of proteins present in the molecular weight marker are indicated to the left and right of their respective gels.

supernatants with expected sizes around, and slightly more than, 30 kDa under reducing conditions (Fig. 1). A Myc-tagged IgG Fc fusion protein was used as a positive control, whereas no product was identified following transfection with the CDM8 vector (Fig. 1). Similarly, Flu-tagged soluble CD94 was expressed and shown to be of the expected size (not shown). Under nonreducing conditions dimer formation was detected for NKG2A, -B, -C, and CD94 extracellular parts (not shown). However, no evident dimer formation was seen for NKG2D and hNKR-P1, the latter of which has been shown to be a homodimer present on a subset of human NK cells [22]. The reason for this discrepancy is at present not known, but it could suggest that residues in the transmembrane and/or cytoplasmic domains are involved in dimer formation, even though this does not seem to be the case for the rat homologue [18]. All lectins seemed to form multimers because reactivity was seen also at higher molecular masses with the anti-Myc tag antibody (not shown).

Soluble NKG2A, -B, and -C interact with soluble CD94 lectin domains

Earlier studies have shown that CD94 interacts with NKG2A and -B on the cell surface of transfected cells [30, 33] and certain NK-like cell lines such as NK92 [31]. Association of NKG2C or -E with CD94 was suggested in a recent paper, but the NKG2 family members that associated with CD94 could not be established because serological reagents distinguishing between the two were not available [33]. Moreover, it was unclear whether or not NKG2D associated with CD94 [33]. In order to directly investigate the association of CD94 with the NKG2 family, we manufactured a Flu-tagged, soluble CD94 and investigated its direct association with Myc-tagged NKG2 subunits and hNKR-P1 following cotransfection experiments in COS cells. Myc-tagged proteins in supernatants from COS cells cotransfected with Flu-tagged sCD94, and Myc-tagged sNKG2A-D and shNKR-P1 were immunoprecipitated on anti-Myc tag Sepharose beads, boiled in sample buffer, and analysed by SDS-PAGE and Western blotting using the anti-Flu tag antibody. Flu-tagged sCD94 was coimmunoprecipitated with Myc-tagged sNKG2A, -B and -C, whereas Myc-tagged sNKG2D, shNKR-P1 and the CD5lMycEγ1 vector product did not coimmunoprecipitate Flu-tagged sCD94 (Fig. 2A). Western blot analysis with the anti-Myc tag antibody on the same beads verified that Myc-tagged sNKG2A-D, shNKR-P1 and IgG Fc proteins were precipitated by the beads (Fig. 2B). The 44 kDa band seen in all lanes in Fig. 2(A,B) is derived from the Ig heavy chain of the anti-Myc tag antibody used for immunoprecipitation.

Soluble CD94, NKG2C and NKG2D C-type lectin domains bind directly to purified HLA class I antigens whereas NKG2A and NKG2B require interaction with CD94 for binding

In order to investigate a possible direct interaction between the soluble lectin domains and HLA class I antigens, we used paramagnetic beads onto which Myc-tagged soluble NKG2A-D, hNKR-P1 or CD94 lectins were absorbed individually, or together with Flu-tagged sCD94 following cotransfection in COS cells. In this latter setting, sCD94 will only bind to the beads if it associates with Myc-tagged sNKG2A-D or shNKR-P1 lectin domains. Paramagnetic beads with bound soluble lectins were incubated with immunoaffinity-purified solubilized HLA class I antigens, whereafter bound HLA class I antigens were detected by flow cytometry using a PE-conjugated anti-HLA class I antibody, or by Western blot analysis using a biotin-conjugated anti-HLA antibody (see Materials and Methods). By

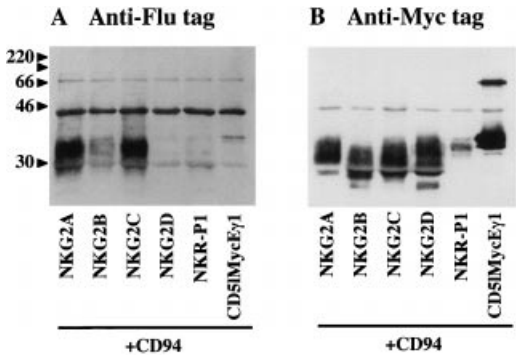

Fig. 2. Western blot analysis of proteins that were anti-Myc tag-immunoprecipitated from supernatants of COS cells cotransfected with sCD94 and sNKG2A, -B, -C, -D, or shNKR-P1. Immunoprecipitated proteins were boiled off 100 μl of anti-Myc tag Sepharose beads, separated on a 10% SDS-polyacrylamide gel under reducing conditions, and transferred to Hybond™-C extra membranes that were probed with anti-Flu (A) or anti-Myc (B) tag antibodies, respectively. The 97 kDa protein in the weight marker is indicated with an arrowhead owing to lack of space.

using the PE-conjugated anti-HLA class I antibody, W6/32, and flow cytometry, we were able to identify a shift in fluorescence following binding of HLA class I antigens to paramagnetic beads carrying soluble NKG2C, -D and CD94 (Fig. 3A). No shift in fluorescence was detected when beads carrying soluble NKG2A, -B and hNKR-P1 were incubated with solubilized HLA class I antigens, indicating that these lectin domains do not bind HLA class I antigens on their own. SDS-PAGE and Western blot analysis of proteins bound to soluble lectin-carrying paramagnetic beads, identified an anti-HLA class I antibody-reactive band of approximately 45 kDa when soluble NKG2C, -D or CD94, but not NKG2A, -B or hNKR-P1, were assayed for binding to solubilized HLA class I antigens (Fig. 4A). Both flow cytometry and Western blot analysis support the interpretation that there is a direct binding of solubilized immunopurified HLA class I antigens by soluble NKG2C, -D or CD94 lectins. The fact that NKG2C, but not NKG2A alone, binds HLA class I antigens is surprising because these molecules are approximately 94% identical in their extracellular domains. Following cotransfection of sNKG2A-D and shNKR-P1 with sCD94, and by using the same assay systems as above, sNKG2A and -B in complex with sCD94 were shown to bind HLA class I antigens (Figs 3B and 4B). It remains to be established whether the binding of HLA class I antigens by NKG2A or -B/CD94 complexes is reflecting the binding activity of CD94 alone, a CD94-modified NKG2A or -B binding activity, or a binding activity partially derived from NKG2A or -B and partially from CD94. It is also not clear whether the binding of NKG2C to HLA class I antigens is potentiated by its association with CD94, or whether the HLA class I antigen specificity of NKG2C/CD94 complexes is different from the specificity of NKG2C and CD94 on their own. The binding of NKG2D to HLA class I antigens in the cotransfection experiment is likely to correspond to a binding effect mediated by NKG2D alone, because sNKG2D could not associate with sCD94 in the coimmunoprecipitation experiments (Fig. 2A). Although not likely, a possible explanation for the lack of HLA class I binding by sNKG2A, -B and shNKR-P1 might be that these lectins bind HLA class I antigens that are not

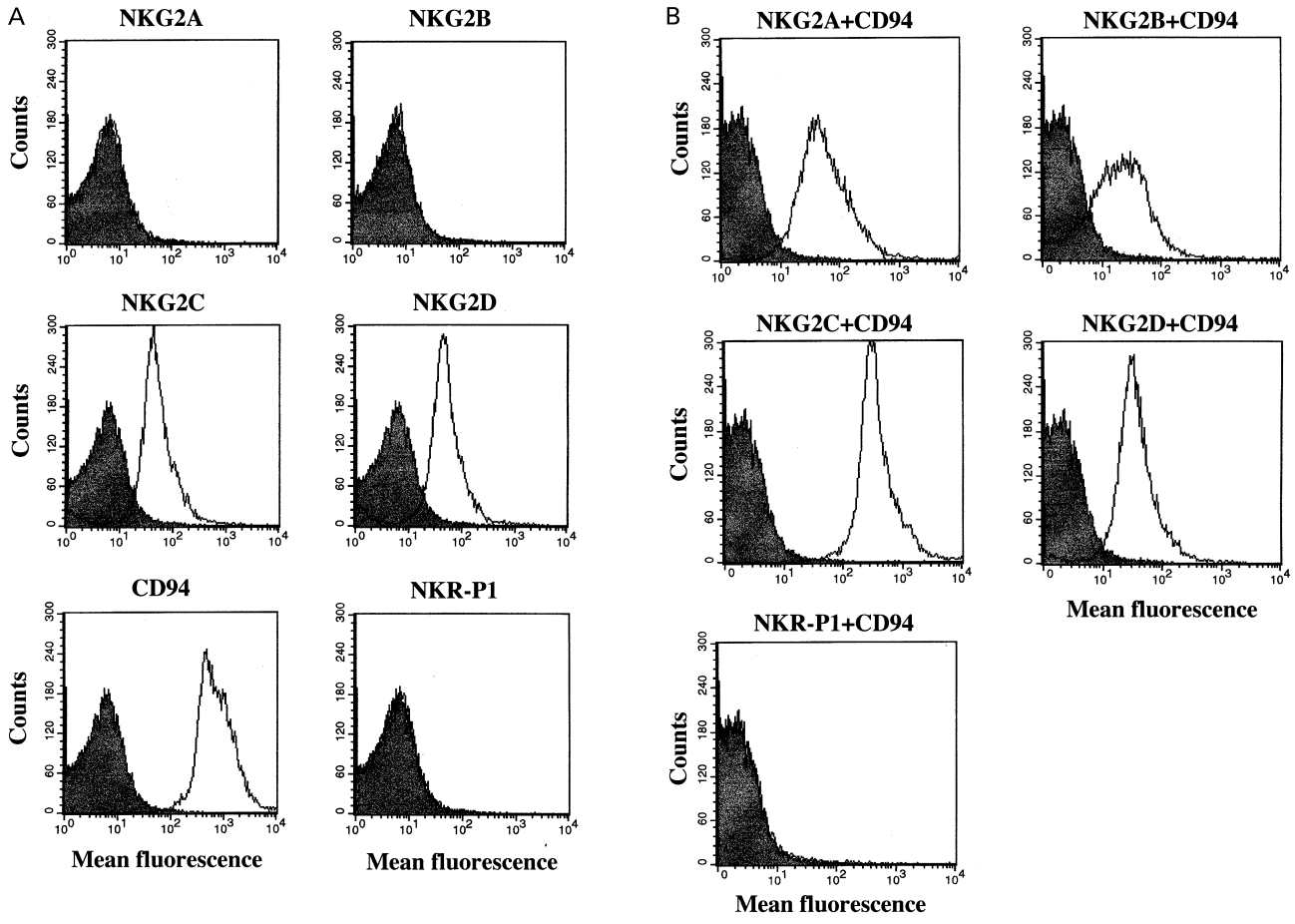

Fig. 3. Flow cytometric analysis of paramagnetic beads carrying sNKG2A, -B, -C, -D, hNKR-P1 and CD94 alone (A), and (B) associated with CD94 (NKG2A, -B, and -C; see Fig. 2A) , following binding of solubilized HLA class I antigens and detection with a PE-conjugated anti-HLA class I antibody. The negative control in (A) (shaded) is paramagnetic beads treated exactly as the lectin-carrying beads, except that they were incubated in supernatant from CDM8-transfected, and not lectin-transfected, COS cells. Similarly, the negative control in (B) (shaded) is beads incubated in supernatants of COS cells transfected with the CD5lMycEγ1 vector together with sCD94.

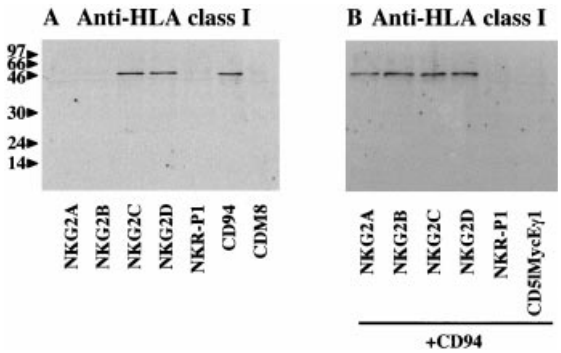

Fig. 4. Western blot analysis of proteins released from paramagnetic beads carrying sNKG2A, -B, -C, -D, hNKR-P1 and CD94 alone (A), and (B) associated with CD94 (NKG2A, -B, and -C; see Fig. 2A), following binding of solubilized HLA class I antigens. Twenty μl of paramagnetic beads were boiled together with 20 μl reducing sample buffer and released proteins were separated on a 15% polyacrylamide gel, transferred to Hybond^{TM}-C extra membranes, and probed with a biotin-labelled anti-HLA class I antibody. Bound antibody was detected with peroxidase-conjugated avidin and chemiluminescence.

represented in the pool of HLA class I antigens used in our experiments. It is also conceivable that we, in agreement with recently published data, are detecting a binding of NK cell lectins to nonclassical HLA class I molecule such as HLA-E [27, 28]. The W6/32 antibody has been shown to bind nonclassical HLA class I antigens [41], and the very distinct W6/32-reactive bands in the Western blot experiment (Fig. 4A,B) indicate little or no size heterogeneity among bound HLA class I antigens, suggesting a single, or a limited number of, HLA class I molecular species.

CONCLUSIONS

The present work shows for the first time that soluble NK lectins, NKG2C, -D and CD94 alone, but not NKG2A and -B, directly bind to solubilized immunoaffinity-purified HLA class I antigens isolated from a pool of platelets obtained from several donors. In agreement with previous studies, we have shown that NKG2A and -B associate with CD94 to form complexes. In our system NKG2C, but not NKG2D, also associates with CD94. This may explain the data published by Lazetic *et al.* where a minimal reactivity of NKG2D- and CD94-coexpressing 293T cells were seen with a rabbit antiserum specific for CD94 associated proteins [33]. Furthermore, the complex of NKG2A, -B or -C with CD94 bound solubilized immunoaffinity-purified HLA class I antigens. For future studies, it will be of importance to characterize the HLA class I antigen specificity of NKG2C, -D and CD94 alone, and to identify a possible shift in specificity following association of NKG2A, -B, or -C with CD94.

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