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Direct binding of a soluble natural killer cell inhibitory receptor to a soluble human leukocyte antigen-Cw4 class I major histocompatibility complex molecule

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ABSTRACT **Natural killer (NK) cells expressing specific p58 NK receptors are inhibited from lysing target cells that express human leukocyte antigen (HLA)-C class I major histocompatibility complex molecules. To investigate the interaction between p58 NK receptors and HLA-Cw4, the extracellular domain of the p58 NK receptor specific for HLA-Cw4 was overexpressed in *Escherichia coli* and refolded from purified inclusion bodies. The refolded NK receptor is a monomer in solution. It interacts specifically with HLA-Cw4, blocking the binding of a p58–Ig fusion protein to HLA-Cw4-expressing cells, but does not block the binding of a p58–Ig fusion protein specific for HLA-Cw3 to HLA-Cw3-expressing cells. The bacterially expressed extracellular domain of HLA-Cw4 heavy chain and β_2-microglobulin were refolded in the presence of a HLA-Cw4-specific peptide. Direct binding between the soluble p58 NK receptor and the soluble HLA-Cw4–peptide complex was observed by native gel electrophoresis. Titration binding assays show that soluble monomeric receptor forms a 1:1 complex with HLA-Cw4, independent of the presence of Zn^{2+}. The formation of complexes between soluble, recombinant molecules indicates that HLA-Cw4 is sufficient for specific ligation by the NK receptor and that neither glycoprotein requires carbohydrate for the interaction.**

Natural killer (NK) cells are a class of lymphocytes that lyse transformed and virally infected cells (1, 2). They provide an important defense mechanism, especially for protection from microorganisms that interfere with the expression of class I major histocompatibility complex (MHC) molecules. The strategy of target cell recognition by NK cells is the reverse of that employed by cytolytic T cells. Whereas cytolytic T cells are activated by recognition of specific class I MHC–peptide complexes on target cells, NK cells are inhibited by recognition of specific class I MHC molecules (reviewed in refs. 3–6). Antigen-specific receptors on T cells mediate cytolytic T-cell activation. Although it is unclear how NK cells are activated to kill target cells, inhibitory receptors that provide specificity in NK recognition have been identified in the mouse and human. Engagement of these receptors by specific class I MHC molecules results in inhibition of the lytic activity of NK cells (7–9).

The murine inhibitory NK receptors, Ly-49, are a polymorphic family of molecules that bind to MHC class I molecules (10, 11). They are disulfide-linked homodimers that belong to the C-type lectin superfamily (12) and recognition by Ly-49 requires the presence of carbohydrates on the class I molecule (13, 14).

Inhibitory receptors on human NK cells are unrelated to Ly-49. They are a polymorphic family of transmembrane glycoproteins of 58 or 70 kDa with two or three extracellular immunoglobulin (Ig)-related domains (15–18). NK clones can be distinguished by their human leukocyte antigen (HLA)-C recognition and antibody binding patterns. Most NK clones that are reactive with mAb GL183 recognize HLA-Cw3 and related alleles (HLA-Cw1, -Cw7, and -Cw8), and those reactive with mAb EB6 recognize HLA-Cw4 and related alleles (HLA-Cw2, -Cw5, and -Cw6) (8, 19, 20). Specific interaction between HLA-C and p58 receptors was suggested by antibody blocking experiments in which either anti-class I or anti-p58 (GL183 and EB6) monoclonal antibodies interfered with class I recognition by NK clones and thus allowed target cell lysis (8, 20, 21). Direct evidence for specific recognition was provided by the binding of soluble Ig fusion proteins of two p58 receptors, one specific for HLA-Cw4 and the other for HLA-Cw3, exclusively to cells transfected with the cognate HLA-C subtype (16).

In this study, we demonstrate direct binding between a recombinant soluble p58 NK receptor and recombinant soluble HLA-Cw4. The soluble NK receptor is monomeric and forms a 1:1 complex with HLA-Cw4. Specific binding is shown to be independent of Zn^{2+}, implying that the zinc requirement for inhibitory action (22) must be at a step other than specific receptor-target binding. Soluble NK receptor is shown to discriminate between HLA-Cw4- and HLA-Cw3-expressing cells. These observations provide evidence that HLA-C is sufficient for specific recognition by the p58 inhibitory receptor and that the interaction is independent of carbohydrate on either protein.

MATERIALS AND METHODS

Gene Sources and Bacterial Strains. The NK receptor p58-cl42 cDNA was contained as a *Sal*I–*Not*I insert in a pSPORT I vector (15). The HLA-Cw4 heavy chain cDNA was contained in the eukaryotic expression vector RSV.5neo (23). The *Escherichia coli* strain XA90 F′ lacI^q (24) was used for cloning. The *E. coli* strain BL21 (DE3) plysS was used for expression of the NK receptor sol-cl42H (amino acids His1-His224), sol-cl42T (amino acids His1-Thr200), and HLA-Cw4 heavy chain (amino acids Gly1-Lys275). β_2m was produced as described (24).

Construction of Expression Plasmids. The protein-coding region of the entire extracellular domain of p58-cl42 (sol-cl42H) was amplified by PCR using *Pfu* DNA polymerase with the 5′-oligonucleotide primer TAGGGCGAATTCTAAGGAGGATATTAAAATGCACGAAGGAGTACACCGTAAACCTTCTCTCCTGGCC and 3′-primer GTTTCAAAGCTTTAGTGCAGGTGACGCGGGTTACCGGTTTT. A shorter fragment of the p58-cl42 (sol-cl42T) was amplified with

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Abbreviations: NK, natural killer; MHC, major histocompatibility complex; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate.

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the same 5′-primer and the 3′-primer, GTTTCAAAGCTTTATGTGACAGAAACAAGCAG. The HLA-Cw4 heavy chain was amplified by PCR with the 5′-primer TAGGGCGAATTCTAAGGAGGATATTAAAATGGGCTCTCACTCTATGCGTTATTTCTCCACA and 3′-primer GTTTCAAAGCTTTACTTCCAACGCAGGGTGAGGGG. The 5′-primer for each construct encoded an ATG start codon. For each construct, the PCR product was doubly digested with *Eco*RI and *Hin*dIII and then ligated into the pLM-1 plasmid. The sequence of each construct was verified by DNA sequencing.

Protein Expression and Preparation of Inclusion Bodies. Plasmids containing the sol-cl42H or sol-cl42T extracellular domain or the HLA-Cw4 heavy chain were transformed into BL21 (DE3) pLysS for expression. Cells carrying expression plasmids were grown from a single colony at 37°C in Luria–Bertani medium containing 100 μg of ampicillin per ml, 34 μg of chloramphenicol per ml, and 0.4% glucose and induced at logarithmic phase with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) to produce protein. The cells were harvested by centrifugation, resuspended in 50 mM Tris·HCl, pH 8.0/25% sucrose/1 mM EDTA, and lysed by freezing and thawing in the presence of 1 mg of lysozyme per ml. The insoluble inclusion bodies were collected, washed extensively to remove contaminating proteins, and dissolved in 8 M urea/25 mM Mes, pH 6.0/10 mM EDTA/1 mM DTT (24).

Refolding by Dilution. The solubilized p58 NK receptor sol-cl42H or sol-cl42T inclusion bodies were diluted into 1 liter of refolding buffer containing 100 mM Tris·HCl, pH 8.3/500 mM L-arginine·HCl/2 mM EDTA/6.4 mM cysteamine (2-mercaptoethylamine)/3.6 mM cystamine/0.1 mM phenylmethylsulfonyl fluoride (PMSF). The final concentration of sol-cl42H was 148.5 μg/liter (6 μM), and the final concentration of sol-cl42T was 133.0 μg/liter (6 μM). The refolding mixture was incubated at 10°C for 72–90 hr. The 1-liter refolding mixture was dialyzed twice, first against 10 liters of 100 mM urea at 4°C for 24 hr and then against 10 liters of 10 mM Tris·HCl, pH 7.5/10 mM Mes/100 mM urea at 4°C for 24 hr.

The solubilized HLA-Cw4 heavy chain and β_2m inclusion bodies, together with an HLA-Cw4 specific peptide, QYDDAVYKL (25), were diluted into 1 liter of refolding buffer containing 100 mM Tris·HCl, pH 8.3/400 mM L-arginine·HCl/2 mM EDTA/5 mM reduced glutathione/0.5 mM oxidized glutathione/0.2 mM PMSF (24). The final concentrations of the HLA-Cw4 heavy chain, β_2m, and peptide were 96 μg/ml (3 μM), 24 μg/ml (2 μM), and 67 μg/ml (60 μM). The refolding mixture was incubated at 10°C for 72–90 hr. The 1-liter refolding mixture was then dialyzed against 10 liters of H_2O at 4°C for 24 hr.

Protein Purification. The refolded p58 NK receptor sol-cl42H (or sol-cl42T) was concentrated and partially purified upon binding to an anion exchange column at pH 7.5, packed with 24 g of the resin diethylaminoethyl cellulose (DE-52). After washing with two column volumes of 10 mM Tris·HCl (pH 7.5), sol-cl42H (or sol-cl42T) was eluted with 10 mM Tris·HCl, pH 7.5/300 mM NaCl and then concentrated with a Centriprep-10 (Amican). The concentrated protein was further purified on a Superdex 200 16/60 fast protein liquid chromatography gel filtration column (Pharmacia) in 10 mM Tris·HCl, pH 7.5/100 mM NaCl. The peak corresponding to 23 kDa was collected. The amount of purified sol-cl42H protein was ≈30 mg, corresponding to a 20% yield of refolding. The amount of purified sol-cl42T protein was ≈50 mg, corresponding to a 37% refolding yield.

The refolded HLA-Cw4 heavy chain and β_2m heterodimer complexed with a peptide was similarly concentrated and partially purified on a DE-52 anion exchange column. The refolded HLA-Cw4 molecule bound to the DE-52 resin at pH 8.0, whereas refolded β_2m was contained in the flowthrough. The column was washed with two column volumes of 20 mM Tris·HCl (pH 8.0), and the HLA-Cw4 protein was then eluted with 20 mM Tris·HCl, pH 8.0/300 mM NaCl. The HLA-Cw4 protein was further separated from any aggregates and contaminating proteins on a Superdex 200 16/60 fast protein liquid chromatography gel filtration column (Pharmacia) in 20 mM Tris·HCl, pH 7.5/150 mM NaCl. A total of 17 mg of purified HLA-Cw4 was obtained, representing a 15% refolding yield.

Competitive Blocking Assay. Transfected 721.221 human B cells expressing HLA-Cw3 or HLA-Cw4 (a gift from J. Gumperz, Stanford University, Palo Alto, CA) were preincubated with 3-fold serial dilutions of soluble sol-cl42H (beginning at 16 μM or 400 μg/ml) for 30 min at 4°C. cl43–Ig was then added to the HLA-Cw3-expressing cells, and cl42–Ig was added to the HLA-Cw4-expressing cells at concentrations predetermined to give equivalent binding activity [i.e., a median fluorescence intensity (MFI) of 20]. Thus, 50 μg of cl43–Ig per ml and 5 μg of cl42–Ig per ml were added for 1 hr at 4°C. The cells were washed and incubated with fluorescein isothiocyanate (FITC) conjugated to goat anti-human antibodies (Jackson ImmunoResearch) for 30 min at 4°C. Fluorescence on a total of 10,000 cells was then analyzed by flow cytometry on a FACScan (Becton Dickinson). The same assay was also carried out using equivalent amounts (15 μg/ml) of the p58–Ig fusion proteins, which corresponded to a MFI of 40.68 for cl42–Ig binding to HLA-Cw4 and a MFI of 10.75 for cl43–Ig binding to HLA-Cw3.

Extinction Coefficient Measurements. The extinction coefficient at 280 nm of the p58 NK receptor sol-cl42H (or sol-cl42T) was determined by measuring the absorbance at 280 nm of the protein and the quantity of the protein using amino acid composition analysis. The concentration of the protein in the sample submitted for amino acid analysis was calculated from the measured quantities of Asx, Glx, Ala, Pro, Leu, and Lys and their percentages by mole in the protein (26). The average extinction coefficient for sol-cl42H obtained from three separate trials of amino acid analyses of the same protein sample was 52921 ± 3848 $M^{-1}\cdot cm^{-1}$. The average extinction coefficient for sol-cl42T was 30197 ± 1728 $M^{-1}\cdot cm^{-1}$.

The molar extinction coefficient at 280 nm of HLA-Cw4 was determined analogously. The average value from three separate trials of amino acid analyses of the protein sample was 110230 ± 13621 $M^{-1}\cdot cm^{-1}$.

Binding Assay of the NK Receptor p58-cl42 and HLA-Cw4. The soluble p58 NK receptor sol-cl42H (or sol-cl42T) and HLA-Cw4 were incubated in a reaction buffer containing 50 mM Tris·HCl, pH 8.0/150 mM NaCl with or without 1 mM 1,10-phenanthroline for 45 min at room temperature. The reaction mixture was then assayed on a 15% native acrylamide gel in 300 mM Tris·HCl, pH 8.5. The native gel running buffer contained 24.8 mM Tris/192 mM glycine.

To determine the effect of zinc on the interaction between soluble p58-cl42 and HLA-Cw4, the binding assay was also carried out in the presence of 0.1 mM $ZnCl_2$ in the reaction mixture, gel mix, and gel running buffer.

Inductively Coupled Plasma Emission Spectroscopic Measurements. The inductively coupled plasma emission spectrometer was calibrated with a 10 parts per billion (ppb) (0.152 μM or 10 μg/liter) and 100 ppb (1.52 μM or 100 μg/liter) zinc standard. The baseline was corrected using distilled H_2O, which contained <1 ppb (15.2 nM or 1 μg/liter) zinc. The specific emission wavelength used to determine the amount of zinc was 206.2 nm. A protein sample containing 259 μM (6.4 mg/ml) of the p58 NK receptor sol-cl42H was diluted with 10 mM Tris·HCl (pH 7.5) buffer to 15.2 μM (0.38 mg/ml). The dilution buffer contained 1.579 ppb (24 nM or 1.579 μg/liter) zinc. The amount of zinc detected in the protein sample was 41.16 ppb (0.63 μM or 41.16 μg/liter) after background correction.

RESULTS AND DISCUSSION

Refolding of the NK Receptor p58-cl42. To study the interaction between the human NK receptors and their class I MHC ligands, we expressed in *E. coli* the entire extracellular domain of a p58 NK receptor specific for HLA-Cw4 (encoded by cDNA p58-cl42; ref. 15) (amino acids His1–His224, denoted sol-cl42H) (Fig. 1*A* and *B*). The receptor protein was produced as inclusion bodies, which were solubilized with 8 M urea. The p58 NK receptor sol-cl42H was refolded to its soluble form upon dilution to remove urea. The molecular weight of the soluble sol-cl42H (24.7 kDa) was confirmed by mass spectroscopy. The purified sol-cl42H exhibited a retention time corresponding to a 23-kDa protein on gel filtration chromatography (Fig. 2*A*). An SDS gel of the eluted protein in the presence of DTT revealed a band with the expected molecular weight of sol-cl42H (Fig. 2*B*, lane 1). The higher mobility of the sol-cl42H protein in the absence of DTT indicated the presence of intramolecular disulfide bonds (Fig. 2*B*, lane 2). Because the most concentrated fraction of the 23-kDa peak on gel filtration contained sol-cl42H at 0.1 mM (2.5 mg/ml), sol-cl42H appears to be monomeric in solution even at this high concentration. In addition, these data show that carbohydrate modification are not essential for refolding of a soluble p58 receptor.

The p58 receptor has an unusually long stem region. To test whether the stem was important for the structure of p58, a shorter version of the NK receptor p58-cl42 (amino acids His1–Thr200, denoted sol-cl42T), which lacks the 24 C-terminal amino acids of the ectodomain, was also refolded and purified (Fig. 1 *A* and *C*). According to its mobility on gel filtration columns (data not shown), the shorter NK receptor sol-cl42T appears to be a monomer. That the shorter NK receptor refolded indicates that the C-terminal stretch of 24 amino acids, which is rich in proline and serine, is possibly a flexible region that does not contribute significantly to the refolding or final structure of the protein.

Soluble NK Receptor p58-cl42 Binds Specifically to HLA-Cw4-Expressing Cells. Soluble p58 NK receptor sol-cl42H bound specifically to HLA-Cw4-expressing target cells as shown by its ability to specifically block binding of a cl42–Ig fusion protein to HLA-Cw4 expressing cells. A purified cl42–Ig fusion protein (the extracellular domain of p58-cl42 fused to the hinge, CH2, and CH3 domains of human IgG1) bound specifically to human 721.221 B cells transfected with the class I allele HLA-Cw4 (.221-Cw4), while a cl43–Ig fusion protein bound specifically to .221-Cw3 transfectants (ref. 16 and this study). The cell binding of p58–Ig fusion proteins was measured by flow cytometry, and an equal level of median fluorescence was achieved with 5 μg of cl42–Ig per ml and 50 μg of cl43–Ig fusion proteins per ml. As shown in Fig. 3*A* and *B*, incubation of .221-Cw4 cells with increasing amounts of soluble sol-cl42H (0–16 μM or 0–400 μg/ml) decreased the binding of cl42–Ig fusion protein to the .221-Cw4 transfectants, but incubation of .221-Cw3 cells with soluble sol-cl42H had no effect on the binding of cl43–Ig fusion protein. Furthermore, the binding of the cl42–Ig fusion protein to .221-Cw4 cells decreased to background levels when excess soluble sol-cl42H was used. When soluble sol-cl42H was used to block the binding of equal amounts of cl42–Ig and cl43–Ig (giving different median fluorescence) to .221-Cw4 and .221-Cw3 target cells, respectively, specific inhibition of the interaction between cl42–Ig and .221-Cw4 cells by soluble sol-cl42H was also observed (Fig. 3*C*). The cell binding assays confirmed the HLA-Cw4 specificity of the NK receptor p58-cl42 and provided evidence that the soluble monomeric NK receptor was able to discriminate among different HLA-C alleles.

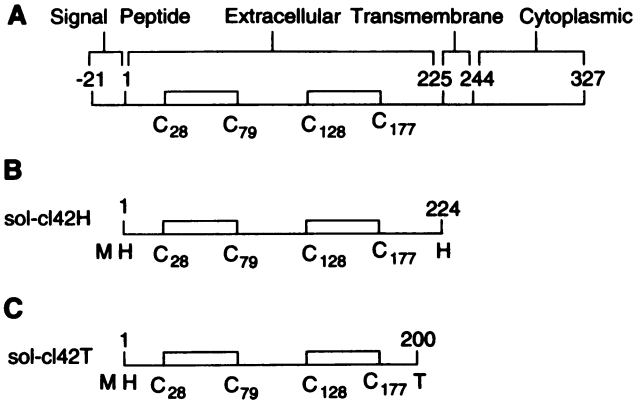

FIG. 1. Schematic diagrams of the sol-cl42H and sol-cl42T constructs for expression in *E. coli*. (*A*) Domain organization of the NK receptor p58-cl42. The NK receptor p58-cl42 is a transmembrane protein consisting of a signal peptide (amino acids −21 to 1), an extracellular domain (amino acids 1–224), a transmembrane region (amino acids 225–243), and a cytoplasmic tail (amino acids 244–327). Cysteines (C) and predicted disulfide bonds are shown. (*B*) The sol-cl42H construct (H denotes the C-terminal amino acid) includes the entire extracellular domain of p58-cl42, with the addition of a methionine (M) at the N terminus. (*C*) The sol-cl42T construct (T denotes the C-terminal threonine residue) encodes the extracellular domain of p58-cl42 and an N-terminal methionine (M) but terminates at Thr200 instead of His224, and it therefore lacks the 24 C-terminal amino acids.

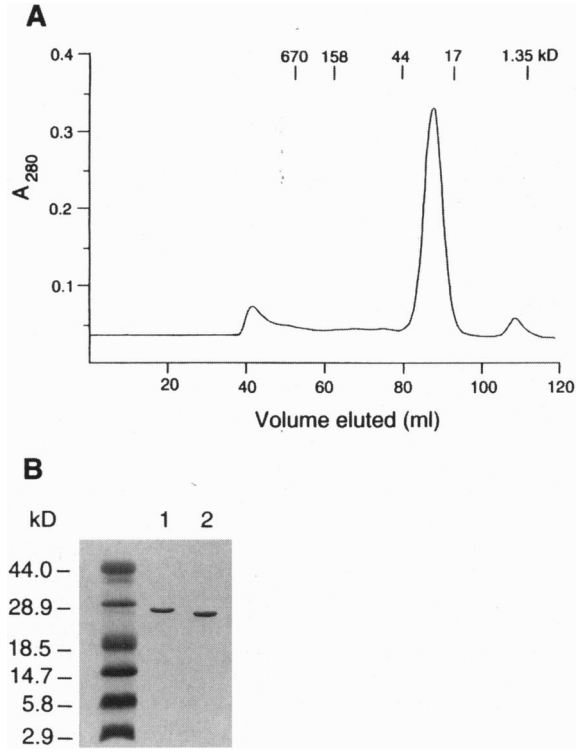

FIG. 2. Purification of the soluble p58 NK receptor sol-cl42H. (*A*) Gel filtration chromatogram of the refolded p58 NK receptor sol-cl42H. The column was calibrated with a mixture of standard proteins with molecular weights as shown. (*B*) SDS/PAGE of the peak in *A* corresponding to 23 kDa, under (lane 1) reducing and (lane 2) nonreducing conditions. The amount of refolded sol-cl42H loaded per lane is 1.2 μg.

Refolding of HLA-Cw4. The extracellular domain of the HLA-Cw4 heavy chain (amino acids Gly1–Lys275) and β_2m were refolded from inclusion bodies in the presence of an HLA-Cw4-binding peptide, QYDDAVYKL (25). The refolded HLA-Cw4 molecule was purified by anion exchange and gel filtration chromatography. The gel filtration chromato-

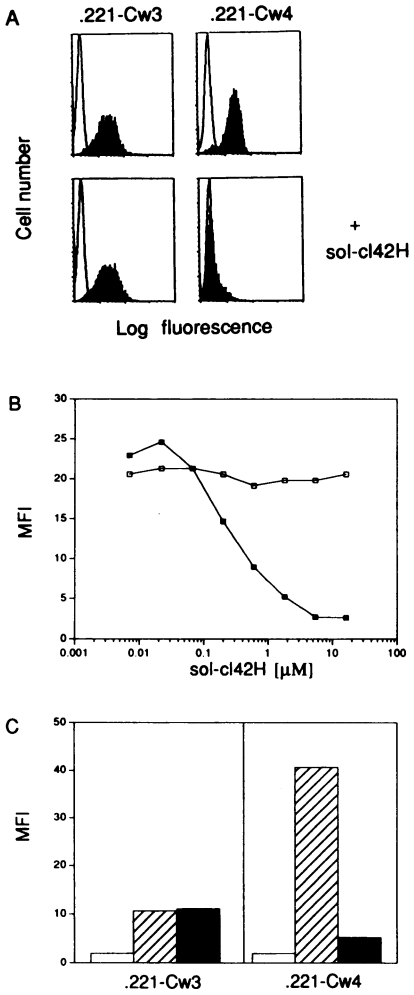

FIG. 3. Soluble sol-cl42H specifically blocks binding of cl42–Ig to HLA-Cw4-expressing cells. (*A*) Transfectants of the human B cell line 721.221 expressing HLA-Cw3 (.221-Cw3) or HLA-Cw4 (.221-Cw4) were preincubated without or with 16 μM (400 μg/ml) sol-cl42H as indicated for 30 min at 4°C. cl43–Ig (50 μg/ml) was then added to the .221-Cw3 cells and cl42–Ig (5 μg/ml) was added to the .221-Cw4 cells for 1 hr at 4°C. The cells were washed, incubated with FITC-conjugated goat anti-human antibody, and analyzed by flow cytometry. The open profiles indicate the level of binding obtained with the FITC-conjugated goat anti-human antibody alone. (*B*) Three-fold serial dilutions (beginning at 16 μM or 400 μg/ml) of soluble sol-cl42H were preincubated with .221-Cw3 cells (open symbols) and .221-Cw4 cells (closed symbols) and assayed for the ability to block binding of cl43–Ig and cl42–Ig, respectively, as described in *A*. (*C*) .221-Cw3 cells (*Left*) and .221-Cw4 cells (*Right*) were preincubated without (hatched) or with (shaded) 16 μM (400 μg/ml) of soluble sol-cl42H. The binding of equivalent amounts 15 μg/ml of either cl43–Ig (*Left*) or cl42–Ig (*Right*) was measured as described in *A*. The open bars indicate the level of binding obtained with the FITC-conjugated goat anti-human antibody alone.

gram contained a major peak with a retention time corresponding to 37 kDa, slightly smaller than the expected molecular weight of soluble HLA-Cw4, 45 kDa (Fig. 4*A*). The 37-kDa peak was shown to correspond to refolded HLA-Cw4 by SDS/PAGE, which demonstrated the presence of both the HLA-Cw4 heavy chain (32 kDa) and β_2m (12 kDa) (Fig. 4*B*). Refolding was not observed in the absence of an HLA-C-specific peptide.

Soluble NK Receptor p58-cl42 Binds Directly to HLA-Cw4 at a 1:1 Ratio. The direct interaction between the soluble NK receptor and HLA-Cw4 was observed using native gel electrophoresis. Both the p58 NK receptor sol-cl42H and sol-cl42T

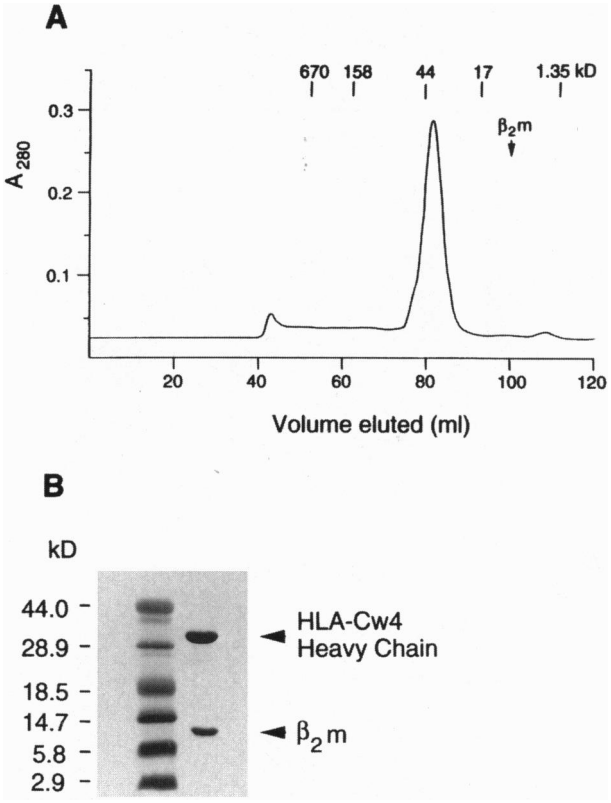

FIG. 4. Purification of soluble HLA-Cw4. (*A*) Gel filtration chromatogram of HLA-Cw4 refolded from heavy chain, β_2m, and the peptide, QYDDAVYKL. The mobility of free β_2m on the gel filtration column is as indicated. The column was calibrated with a mixture of standard proteins with molecular weights as shown. (*B*) SDS/PAGE of the peak in *A* corresponding to 37 kDa. The amount of refolded HLA-Cw4 loaded on the gel is 2.8 μg.

formed complexes with HLA-Cw4 (Fig. 5 *A* and *B*). Free NK receptor sol-cl42H (or sol-cl42T) appeared as two bands on native gel, in contrast to the single band on SDS gel, indicating the presence of charge heterogeneity in the purified sol-cl42H (or sol-cl42T) protein. The observation of novel bands in the mixture of HLA-Cw4 and sol-cl42H (or sol-cl42T) demonstrates formation of a complex between the soluble molecules. Both bands of the NK receptor sol-cl42H (or sol-cl42T) could be gel-shifted to form complexes with HLA-Cw4 [Fig. 5 *A* and *B*, complex formation at 1:1 and 1:2 ratios of sol-cl42H (or sol-cl42T) to HLA-Cw4]. Neither sol-cl42H nor sol-cl42T could be gel shifted by soluble HLA-A2 or HLA-B27 to yield complex bands (data not shown), indicating that the *in vitro* binding observed between the p58 NK receptor and HLA-Cw4 was specific.

The stoichiometry of the sol-cl42H (or sol-cl42T)–HLA-Cw4 complex was determined by titration binding assays on native gel electrophoresis. To determine the concentrations of sol-cl42H (or sol-cl42T) and HLA-Cw4, their molar extinction coefficients were calculated from UV absorbance measured at 280 nm, and protein quantity was measured by amino acid composition analysis. For HLA-Cw4, the measured molar extinction coefficient was 110230 ± 13621 $M^{-1}\cdot cm^{-1}$, which agreed with the predicted extinction coefficient, 101210 $M^{-1}\cdot cm^{-1}$ (27). The high extinction coefficient of HLA-Cw4 is consistent with the high content of tryptophan (3.4%) and tyrosine (5.5%). For the NK receptor sol-cl42H, the measured extinction coefficient was 52921 ± 3948 $M^{-1}\cdot cm^{-1}$, almost twice the predicted value, 27550 $M^{-1}\cdot cm^{-1}$. When the NK receptor sol-cl42H and HLA-Cw4 were incubated at a 1:1 ratio, the receptor protein and HLA-Cw4 were completely

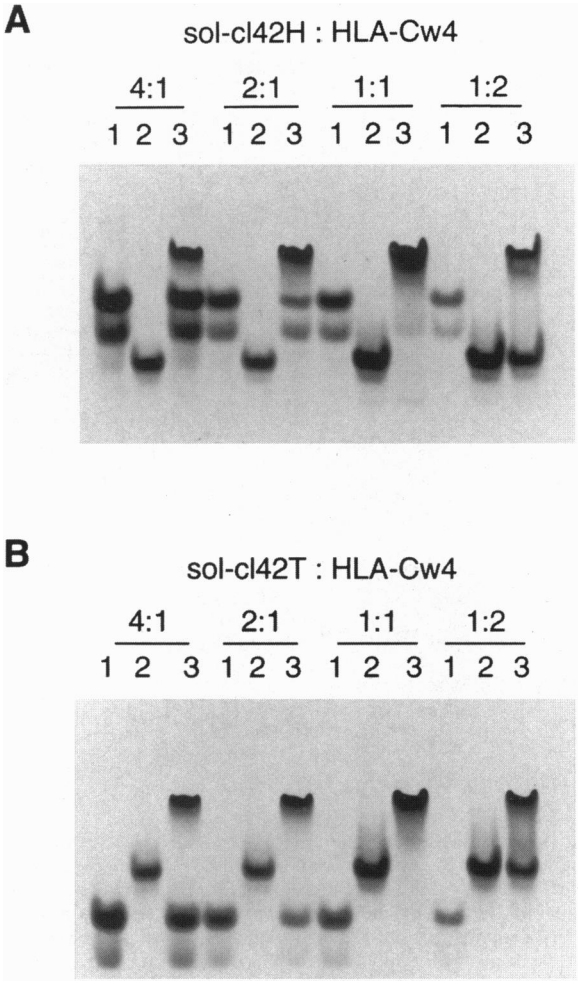

FIG. 5. Soluble NK receptor p58-cl42 binds to HLA-Cw4. (*A*) Native gel electrophoresis (15%) of the binding between sol-cl42H and HLA-Cw4 at molar ratios 4:1, 2:1, 1:1, and 1:2 of sol-cl42H to HLA-Cw4. For each binding ratio, lane 1 is sol-cl42H control, lane 2 is HLA-Cw4 control, and lane 3 is a binding assay of the same amount of sol-cl42H and HLA-Cw4 as in the control lanes. The concentration of HLA-Cw4 is 8.3 μM at ratios 4:1 and 2:1 and 16.7 μM at ratios 1:1 and 1:2. (*B*) Native gel electrophoresis (15%) of the binding between sol-cl42T and HLA-Cw4 at molar ratios 4:1, 2:1, 1:1, and 1:2 of sol-cl42T to HLA-Cw4. For each binding ratio, lane 1 is sol-cl42T control, lane 2 is HLA-Cw4 control, and lane 3 is a binding assay of the same amount of sol-cl42T and HLA-Cw4 as in the control lanes. Soluble sol-cl42T is more negatively charged and lower in molecular weight than sol-cl42H, therefore it has a greater mobility on a native gel than sol-cl42H. The concentration of HLA-Cw4 is 8.3 μM at ratios 4:1 and 2:1, and 16.7 μM at ratios 1:1 and 1:2.

shifted into the complex band, indicating a 1:1 binding stoichiometry of NK receptor and HLA-Cw4 (Fig. 5*A*).

The 1:1 binding stoichiometry of the NK receptor and HLA-Cw4 complex was confirmed by a titration binding assay using the shorter NK receptor sol-cl42T. The measured extinction coefficient of sol-cl42T was 30197 $\pm$ 1728 $M^{-1}\cdot cm^{-1}$, about 1.4 times the predicted value, 21860 $M^{-1}\cdot cm^{-1}$. Both the shorter NK receptor and HLA-Cw4 were completely gel-shifted to form the complex at a 1:1 binding ratio (Fig. 5*B*). The 1:1 binding stoichiometry observed by native gel electrophoresis is also consistent with the formation of 2:2 (or higher order) complexes between dimers (or multimers) of sol-cl42 and soluble HLA-Cw4 molecules. To address this possibility, both long and short NK receptors were mixed in a single titration with HLA-Cw4. No novel bands were observed (data not shown), as would have been expected for a dimeric interaction of two NK receptors of different length with HLA-C. Therefore, our data show that the soluble p58 NK receptor forms 1:1 complexes with soluble HLA-Cw4 molecules.

The 1:1 binding stoichiometry is consistent with signaling mechanisms that involve changes in the conformation of the NK receptor upon binding to class I MHC molecules or increases in the local density of NK receptor–MHC class I complexes. This 1:1 stoichiometry also implies that specific binding to HLA-C molecules by p58 NK receptors is not dependent on the formation of homodimers or heterodimers of p58 molecules.

Soluble p58 NK Receptor Binding is Independent of Zinc Ions. The presence of two zinc-binding motif (HEXXH) sequences, HEGVH and HFLLH, in the extracellular domain of the p58 NK receptors suggested a role for zinc in the structure and function of p58 (15, 22). A p58 NK receptor solubilized from an NK cell line bound to a zinc affinity column (22). Furthermore, the addition of a zinc chelator, 1,10-phenanthroline, at 2 mM or 4 mM restored lysis by NK clones of the target cells, which were normally protected by expression of HLA-C (22).

The effect of zinc on the specific interaction between p58 and HLA-C was examined using soluble p58 NK receptor sol-cl42H and HLA-Cw4. The gel-shift binding of sol-cl42H with HLA-Cw4 was observed in the presence of 1 mM 1,10-phenanthroline, suggesting that zinc had no effect on the binding of the NK receptor sol-cl42H to HLA-Cw4. Zinc did not affect the oligomerization state of the NK receptor sol-cl42H, because sol-cl42H remained monomeric on the gel filtration column at 0.1 mM (2.5 mg/ml) and in the presence of 0.1 mM $ZnCl_2$. The possibility that zinc was already bound to the NK receptor during refolding and purification was tested by a direct measurement of the amount of zinc contained in the protein sample using inductively coupled plasma emission spectroscopy. The protein sample contained a substoichiometric amount of zinc (1/24 of the amount of protein), indicating that zinc was not stably associated with the soluble p58 receptor. In addition, the p58 NK receptor sol-cl42H could be refolded in the presence of 2 mM EDTA and 1 mM 1,10-phenanthroline. This refolded NK receptor also bound to HLA-Cw4 (data not shown). Thus, zinc does not appear to be an integral part of the structure of this soluble, recombinant p58 NK receptor, nor is it required for the specific binding of HLA-C by the receptor. These observations suggest that the requirement for zinc is subsequent to the binding of p58 receptor to HLA-C.

Conclusions. We have expressed in *E. coli* the extracellular domain of the NK receptor p58-cl42. Soluble p58-cl42 can be obtained by refolding. The HLA-Cw4 specificity of the NK receptor p58-cl42 is demonstrated by its ability to competitively block the binding of a cl42–Ig fusion protein to HLA-Cw4-expressing cells, but not the binding of a cl43–Ig fusion protein to HLA-Cw3-expressing cells. The class I MHC molecule HLA-Cw4 has also been reconstituted from bacterially expressed heavy chain and β_2m. Soluble p58-cl42 binds to soluble HLA-Cw4 at a 1:1 ratio, demonstrating that the recognition of HLA-C-expressing target cells by NK cells is mediated through the direct binding between inhibitory p58 NK receptors and HLA-C molecules. Although there are five potential N-linked glycosylation sites in the extracellular domain of p58-cl42 and one in the extracellular domain of HLA-Cw4 heavy chain, the binding of bacterially produced p58-cl42 to HLA-Cw4 indicates that carbohydrate modifications of the molecules are not required for this interaction.

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1. Malnati, M. S., Lusso, P., Ciccone, E., Moretta, A., Moretta, L. & Long, E. O. (1993) *J. Exp. Med.* **178,** 961–969.
2. Brutkiewicz, R. R. & Welsh, R. M. (1995) *J. Virol.* **69,** 3967–3971.
3. Yokoyama, W. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3081–3085.
4. Leibson, P. J. (1995) *Immunity* **3,** 5–8.
5. Raulet, D. H. & Held, W. (1995) *Cell* **82,** 697–700.
6. Gumperz, J. E. & Parham, P. (1995) *Nature (London)* **378,** 245–248.
7. Karlhofer, F. M., Ribaudo, R. K. & Yokoyama, W. M. (1992) *Nature (London)* **358,** 66–70.
8. Moretta, A., Vitale, M., Bottino, C., Orengo, A. M., Morelli, L., Augugliaro, R., Barbaresi, M., Ciccone, E. & Moretta, L. (1993) *J. Exp. Med.* **178,** 597–604.
9. Litwin, V., Gumperz, J., Parham, P., Phillips, J. H. & Lanier, L. L. (1994) *J. Exp. Med.* **180,** 537–543.
10. Kane, K. P. (1994) *J. Exp. Med.* **179,** 1011–1015.
11. Daniels, B. F., Karlhofer, F. M., Seaman, W. E. & Yokoyama, W. M. (1994a) *J. Exp. Med.* **180,** 687–692.
12. Yokoyama, W. M. & Seaman, W. E. (1993) *Annu. Rev. Immunol.* **11,** 613–635.
13. Daniels, B. F., Nakamura, M. C., Rosen, S. D., Yokoyama, W. M. & Seaman, W. E. (1994b) *Immunity* **1,** 785–792.
14. Brennan, J., Takei, F., Wong, S. & Mager, D. L. (1995) *J. Biol. Chem.* **270,** 9691–9694.
15. Wagtmann, N., Biassoni, R., Cantoni, C., Verdiani, S., Malnati, M., Vitale, M., Bottino, C., Moretta, L., Moretta, A. & Long, E. O. (1995a) *Immunity* **2,** 439–449.
16. Wagtmann, N., Rajagopalan, S., Winter, C. C., Peruzzi, M. & Long, E. O. (1995b) *Immunity* **3,** 801–809.
17. Colonna, M. & Samaridis, J. (1995) *Science* **268,** 405–408.
18. D'Andrea, A., Chang, C., Franz-Bacon, K., McClanahan, T., Phillips, J. H. & Lanier, L. L. (1995) *J. Immunol.* **155,** 2306–2310.
19. Colonna, M., Borsellino, G., Falco, M., Ferrara, G. B. & Strominger, J. L. (1993) *Proc. Natil. Acad. Sci. USA* **90,** 12000–12004.
20. Vitale, M., Sivori, S., Pende, D., Moretta, L. & Moretta, A. (1995) *Proc. Natil. Acad. Sci. USA* **92,** 3536–3540.
21. Ciccone, E., Pende, D., Vitale, M., Nanni, L., Di Donato, C., Bottino, C., Morelli, L., Viale, O., Amoroso, A., Moretta, A. & Moretta, L. (1994) *Eur. J. Immunol.* **24,** 1003–1006.
22. Rajagopalan, S., Winter, C. C., Wagtmann, N. & Long, E. O. (1995) *J. Immunol.* **155,** 4143–4146.
23. Biassoni, R., Falco, M., Cambiaggi, A., Costa, P., Verdiani, S., Pende, D., Conte, R., Di Donato, C., Parham, P. & Moretta, L. (1995) *J. Exp. Med.* **82,** 605–609.
24. Garboczi, D. N., Hung, D. T. & Wiley, D. C. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 3429–3433.
25. Sidney, J., del Guercio, M., Southwood, S., Engelhard, V. H., Appella, E., Rammensee, H., Falk, K., RÖtzschke, O., Takiguchi, M., Kubo, R. T., Grey, H. M. & Sette, A. (1995) *J. Immunol.* **154,** 247–259.
26. Pantoliano, M. W., Horlick, R. A., Springer, B. A., Van Dyk, D. E., Tobery, T., Wetmore, D. R., Lear, J. D., Nahapetian, A. T., Bradley, J. D. & Sisk, W. P. (1994) *Biochemistry* **33,** 10229–10248.
27. Edelhoch, H. (1967) *Biochemistry* **6,** 1948–1954.