The Natural Killer Cell Receptor Specific for HLA-A Allotypes: A Novel Member of the p58/p70 Family of Inhibitory Receptors That Is Characterized by Three Immunoglobulin-like Domains and Is Expressed as a 140-kD Disulphide-linked Dimer

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Summary

Human natural killer (NK) cells express inhibitory receptors that are specific for different groups of HLA-C or HLA-B alleles. The majority of these receptors belong to the immunoglobulin (Ig) superfamily and are characterized by two or three extracellular Ig-like domains. Here we describe a novel inhibitory NK receptor that is specific for a group of HLA-A alleles. The HLA-A3–specific NK cell clone DP7 has been used for mice immunization. Two mAbs, termed Q66 and Q241, bound to the immunizing clone and stained only a subset of NK cell populations or clones. Among Q66 mAb-reactive clones, we further selected those that did not express any of the previously identified HLA-class I–specific NK receptors. These clones did not lyse HLA-A3$^+$ (or -A11$^+$) target cells, but lysis of these targets could be detected in the presence of Q66 or Q241 mAbs. On the other hand, target cells expressing other HLA-A alleles, including -A1, -A2, and -A24, were efficiently lysed. Moreover, none of the HLA-C or HLA-B alleles that were tested exerted a protective effect. Q66$^+$, but not Q66$^-$ NK cell clones, expressed messenger RNA coding for a novel 3 Ig domain protein homologous to the HLA-C (p58) and HLA-B (p70) receptors. The corresponding cDNA (cl.1.1) was used to generate transient and stable transfectants in COS7 and NIH3T3 cell lines, respectively. Both types of transfectants were specifically stained by Q66 and Q241 mAbs. Since the cytoplasmic tail of Q66-reactive molecules was at least 11 amino acid longer than the other known p58/p70 molecules, we could generate an antiserum specific for the COOH-terminus of Q66-reactive molecules, termed PGP-3. PGP-3 immunoprecipitated, only from Q66$^+$ NK cells, molecules displaying a molecular mass of 140 kD, under nonreducing conditions, which resolved, under reducing conditions, in a 70-kD band. Thus, differently from the other p58/p70 receptors, Q66-reactive molecules appear to be expressed as disulphide-linked dimers and were thus termed p140. The comparative analysis of the amino acid sequences of p58, p70, and p140 molecules revealed the existence of two cysteins proximal to the transmembrane region, only in the amino acid sequence of p140 molecules.

NK cells are thought to play a relevant role in the host defenses because of their ability to kill certain tumor or virally infected cells (1). The molecular mechanisms that explain why NK cells do not kill indiscriminately but spare normal (self) cells have recently been elucidated. It reflects the expression of different specialized receptors that recognize allelic forms of MHC class I molecules expressed on normal cells. This interaction leads to the generation of an inhibitory signal that prevents target cell lysis (2–6). Accordingly, the lack of expression of one or more class I alleles (as it may occur in tumor or virally infected cells), leads to NK-mediated target cell lysis. So far, different

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types of receptors specific for distinct groups of HLA-C (p58 molecules) or HLA-B (p70/NKB1 molecules) alleles have been identified (7–10). Thus, p58.1 molecules reactive with the EB6 mAb recognized a group of HLA-C alleles (HLA-Cw2, -Cw4, -Cw5, and -Cw6), characterized by the amino acid residues N77/K80 (4). p58.2 (reactive with GL183 mAb) recognized HLA-Cw1, Cw3, Cw7, and Cw8 alleles characterized by S77/N80 (4). p70/NKB1 recognized a group of HLA-B alleles belonging to the Bw4 supertypic specificity (e.g., HLA-B2705, -B51, -B58, and -B44) (9, 10). Cloning of these receptors revealed new members of the Ig superfamily, characterized by two (p58) or three (p70/NKB1) Ig-like domains in their extracellular portions and by a long cytoplasmic tail associated with a nonpolar transmembrane (11–13). The cytoplasmic tail is characterized by an immuno tyrosine-based activatory motif (ITAM)[1]-like sequence in which two YXXL amino acid motifs are spaced by 26 amino acids (instead of six to eight typical of ITAMs of CD3 molecules) (14). CD94, another putative receptor involved in HLA class I recognition, displays a different structure. Similar to Ly49, the mouse receptor for MHC class I molecules, it belongs to the type 2 transmembrane proteins containing a C-type lectin domain (15, 16). So far, only receptors for HLA-C or HLA-B alleles have been identified; however, evidence for an NK-mediated recognition of HLA-A alleles has been provided both in fresh (17) and cloned NK cells (18).

In the present paper, we selected two mAbs that identify a receptor recognizing HLA-A3, but not -A1, -A2, and -A24 alleles. Similar to other HLA class I–specific NK receptors, this receptor is expressed on a subset of NK cells, and it inhibits the NK-mediated cytolysis upon interaction with its specific ligand. Different from the other NK receptors, Q66 is expressed at the NK cell surface as a disulphide-linked dimer of 140 kD (thus termed p140). In addition, molecular analysis revealed a new member of the Ig superfamily, characterized by three extracellular Ig-like domains and a nonpolar transmembrane portion associated with a 95–amino acid cytoplasmic tail containing an ITAM-like motif.

Materials and Methods

NK Clones and Populations. PBL derived from healthy donors were isolated on Ficoll-Hypaque gradients, and cells were then incubated with a mixture of Leu4, Leu3a, and Leu2a mAbs for 30 min at 4°C, followed by treatment with goat anti–mouse–coated Dynabeads (Dynal, Oslo, Norway) for 30 min at 4°C. Next, after immunomagnetic depletion, $CD3^-4^-8^-$ cells were cloned under limiting dilution conditions in the presence of irradiated feeder cells, 0.1% (vol/vol) PHA (Gibco Ltd., Paisley, Scotland), and exogenous IL2 (rIL-2; Cetus Corp., Emeryville, CA), as previously described (19). $CD3^-56^+16^+$ polyclonally activated NK cell populations were obtained plating 10^4 $CD3^-4^-8^-$ cells per well at the same culture conditions.

Production of mAbs. 5-wk-old male BALB/c mice were immunized with the NK cell clone DP7 (surface phenotype: $CD3^-$ $CD16^+CD56^+GL183^-EB6^-Z27^-$), as previously described (7). The screening of hybridoma supernatants was based on the ability to reconstitute the cytolytic function of the DP7 clone against the C1R/A3 transfectant used as ^{51}Cr-labeled target cells in a 4-h ^{51}Cr release assay, as previously described in detail (7). According to this screening procedure, two hybridomas termed Q66 and Q241 were selected and further subcloned in limiting dilution. Both were of the IgM isotype.

mAbs and Flow Cytometry. In this study, the following mAbs were used: Leu4 (IgG_1 anti-CD3), Leu3a (IgG_1 anti-CD4), and Leu2a (IgG_1 anti-CD8), purchased by Becton Dickinson & Co. (Mountain View, CA); GL183 (IgG_1 anti-p58.2), EB6 (IgG_1 anti-p58.1), XA-141 (IgM anti-p58.1), c127 (IgG_1 anti-CD16), c218 (IgG_1 anti-CD56), A6-136 (IgM anti–HLA class I), Z27 (IgG_1 anti-p70/NKB1), XA185 (IgG_1 anti-CD94), and A6-220 (IgM anti-CD56) were obtained in our lab (7–9, 20–22).

Analysis of PBL or polyclonally activated NK cell populations for the distribution of the surface antigen recognized by the Q66 mAb, was performed using two-color fluorescence cytofluorimetric analysis (FACSort®, Becton Dickinson), as previously described (8). Cells were stained with Q66 mAb, followed by PE-conjugated goat anti–mouse IgM antibodies (Southern Biotechnology Associates, Birmingham, AL), and the other mAb, either Leu4, c127, GL183 and EB6, or Z27 antibodies, followed by appropriate anti–IgG_1-FITC antibodies.

Modulation of Surface Molecules. Flat-bottommed plates (24 wells/plate; Costar Corp., Cambridge, MA) were coated with 10 μg/ml (1 ml) XA-141 mAb (anti-p58) during overnight incubation at 4°C, as previously described (20). Cloned NK cells (10^6) were added to coated or uncoated wells and incubated for 18 h at 37°C. Cells were then washed twice and analyzed for indirect immunofluorescence on a flow cytometer by a FACSort®.

Cytolytic Assay. The cytolytic activity of cloned NK cells was measured in a 4-h ^{51}Cr-release assay in which effector cells were tested against the C1R B-LCL human cell line (5) (HLA-A^-, -$B35^{low}$; -$Cw4^+$) and C1R transfectants expressing HLA-A1, -A2, -A3, -A24, -B51, -B7, and -B27 (kindly provided by P. Cresswell, Yale University, New Haven, CT; B. Biddison, National Institutes of Health, Bethesda, MD and J.A. Lopez De Castro, Fundacion Yimenez Diaz, Madrid, Spain). Other target cells used in these studies were represented by the B-EBV cell lines HOM2 (homozygous haplotype: HLA-A3; -B27; -Cw1), SP0010 (homozygous haplotype: HLA-A2; -B44; -Cw5), and WT100BIS (homozygous haplotype: HLA-A11; -B35; -Cw4), provided by Dr. G.B. Ferrara (Istituto Scientifico Tumori, Genova, Italy) and the B-EBV A51 (haplotype: HLA-A2; -B18, 15; -Cw3,7) derived from a normal HLA-typed individual (Table 1) (4). All these target cells were used at 5×10^3/well at the final E/T ratio of 4:1. The percent of specific lysis was determined as previously described (4). mAbs were added at the onset of the cytolytic assay before adding target cells. The concentrations of Q66 and A6-136 mAbs used in the various assays were 2 μg/ml and 500 ng/ml, respectively.

cDNA Library Construction and Size Fractionation. RNA was prepared from $CD3^-16^+$ NK cells by the guanidinium thiocyanate/CsCl method, and cDNA was synthesized from 8 μg of the poly(A)$^+$ fraction by the method of Gubler and Hoffman (26). The cDNA was inserted into the CDM8 vector using non–self-complementary BstXI adapters (26), and the ligated DNA was transformed into competent *Escherichia coli* MC 1061/p3. 100 μg of the amplified library were then linearized with NotI and size

[1] *Abbreviations used in this paper:* ITAM, immuno tyrosine-based activatory motif; ORF, open reading frame; RACE, rapid amplification of cDNA ends.

Table 1. *HLA Class I Typing of the B-EBV Cell Lines Used as Target Cells*

B-EBV cell line	HLA classs I haplotype		
	Locus A	Locus B	Locus C
HOM2*	A3	B27 (Bw4)‡	Cw1(S77/N80)§
WT100BIS*	A11	B35 (Bw6)	Cw4 (N77/K80)
SP0010*	A2	B44 (Bw4)	Cw5 (N77/K80)
A51	A2,A2	B18,B15, (Bw6)	Cw3,7 (S77/N80)

*Homozygous B-EBV cell line.
‡Indicates the supertypic specificity of the expressed HLA-B allele.
§Indicates the amino acid residues at positions 77 and 80 of the expressed HLA-C allele.

fractionated using a 0.8% agarose gel. 12 different fractions were religated after Gene-Clean (Bio 101, La Jolla, CA) purification and transformed into MC 1061/p3 by electroporation at 2,500 V, 200 Ω, and 25 μF (26) using a Bio-Rad apparatus (Bio-Rad Laboratories srl, Milano, Italy).

Isolation of cDNA Clones Encoding 3 Ig Domain NK Receptors. Replicas were made on 137-mm nylon filters (MSI, Westboro, MA) and prepared for hybridization as described (26). The filters were hybridized with a 403-bp cDNA probe (nucleotides 88–491 of 183cl6 sequence). Different recombinant cDNA clones were identified, subcloned, and plasmid DNA was isolated as described (27). The 5′-end nucleotide sequences of 17.1 and 8.11 cDNAs were checked by rapid amplification of cDNA ends (RACE) (28).

Amplification of Specific cDNAs from NK Cell Clones. Total RNA was extracted using RNAzol (Cinna/Biotecx, Houston, TX) by standard procedure. cDNA was obtained by reverse transcription reaction using oligo dT priming. Primers used for cDNA amplification were 5′ ACGTTCCCATCTTCCA(TC)G (backward) and 5′ GCT(AG)CAGGACAAGGTCAC (forward). The set of primers (0.5 μM) was used in a 30 cycles PCR including 15 cycles at 30 s 94°C, 30 s 65°C, 30 s 72°C, and 15 cycles at 30 s 94°C, 30 s 60°C, 30 s 72°C followed by an extension of 7 min at 72°C. cDNA fragments were subcloned into pCRII vector (Invitrogen, San Diego, CA) and sequenced.

Dot Blot Hybridization. Amplified fragments were denaturated in 0.45 N NaOH, spotted onto nylon membrane (GeneScreen Plus NEN DUPONT, Boston, MA), and hybridized with the following ^{32}P-labeled oligonucleotides: 5′ CTA CAG ATG TTA TGG TTC TGT T (probe Q) and 5′ TAC AGA TGC TAC GGT TCT GT (probe Z). Hybridization was carried out for 4 h at 56°C in 0.1% SDS, 5× Denhardt's solution, and 4× SSPE (27) (probe Q) or 5× SSPE (probe Z). Membranes were washed in 0.1% SDS and 0.1× SSPE for 10 min at room temperature and for 10 min at 42°C (probe Z) or 44°C (probe Q).

Isolation of Full-length cDNAs. The sequences of the forward primers used to obtain complete open reading frame (ORF) amplification products were 5′ CATGT(CT)GCTCA(CT)GGTCGTC (Ig3Up forward), and 5′ GGTTTTGAGACAGGGCTG (Ig3Down backward). The PCR cycling condition used was 15 cycles at 20 s 94°C, 30 s 65°C, 30 s 72°C, and 15 cycles at 20 s 94°C, 30 s 60°C, 30 s 72°C, followed by an extension of 7 min at 72°C. The 5′ end nucleotide sequences of cl.17.1, cl.1.1, and cl.AMC5 cDNAs were checked by RACE (28). cDNA fragments were subcloned into pCR3 vector (Invitrogen) and sequenced.

Transient Transfectants. The monkey COS7 cell line (5 × 10^5/plate) was transfected by the DEAE-dextran method with pCR3-cl.1.1 and pCR3-AMC5 cDNA constructs. In particular, 5 × 10^5 COS7 cells were incubated in DMEM/0.1 mM chloroquine phosphate/0.5 mg/ml DEAE-dextran in the presence of 10 μg of plasmid DNA at 37°C. After 3 h, the transfection mixture was replaced by DMEM/10% FCS. After 48 h, transfectants were trypsinized, stained with Q66, Q241, Z27, EB6, and GL183 mAbs, followed by a PE-conjugated goat antibodies to mouse IgM or IgG_1 and analyzed by a FACSort®.

Stable Transfectants. Subconfluent NIH3T3 cells were transfected by the calcium phosphate technique with the cDNA construct pCR3-cl.1.1, as already described (27). 48 h after transfection, the cells were selected in G418 (1 mg/ml). 2 wk later, cells were analyzed for cell-surface expression with Q66 and Q241 mAbs, sorted, and subcloned.

Western Blot Analysis. Cells were lysed for 30 min on ice in a buffer containing 1% NP40, 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 1 mM PMSF, 10 mM iodoacetamide. Lysates were run on a 8.5% polyacrylamide gel under reducing (5% β-mercaptoethanol) or nonreducing conditions. Gel was blotted in transfer buffer (25 mM Tris-HCl, pH 8.3/0.2 M glycine in 20% vol/vol methanol) on a polyvinyldifluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA) at 125 mA for 1 h. The blot was saturated with TBS (0.15 M NaCl/20 mM Tris-HCl, pH 7.5)/3% nonfat dried milk overnight at 4°C. The blot was incubated with PGP3 antiserum (1:200 dilution) for 2 h followed by a 1-h incubation with a 1:10,000 dilution of a goat anti–rabbit IgG conjugated to alkaline phosphatase (Tropix Inc., Bedford, MA). Chemioluminescent detection was performed using CSPD substrate (Tropix) following the manufacturer's instructions. X-ray films were exposed at room temperature for some minutes to detect the chemiluminescence.

PGP3 Polyclonal Antiserum. A 2.5-kg HY/Cr male rabbit (Charles River Laboratories, Wilmington, MA) was immunized with 100 μg/100 μl of the 15–amino acid peptide VSCPRAPQSGLEGVF (MedProbe, Oslo, Norway) conjugated with KLH. Four treatments were performed at 1-wk intervals; the first one was in association with 100 μl CFA, the other ones in association with 100 μl IFA. After 1 wk from the last treatment, 10 ml of blood was drawn and the serum was tested and titered by ELISA against the immunizing peptide and irrelevant ones. Other immunizations followed at 2-wk intervals.

ELISA. The peptide used for immunization and two nonrelevant peptides were diluted in 10 mM phosphate buffer, pH 7.0 (5 μl/ml and attached to a polyvinylchloride plate (50 μl/well) overnight at 4°C. The plate was saturated with 3% BSA/PBS and incubated with scalar dilutions of preimmune serum or PGP3 antiserum, followed by a horseradish peroxidase–labeled goat anti-rabbit Ig antibody (1:5,000). Positivity was detected after incubation with ABTS substrate (Boehringer Mannheim, GmbH, Mannheim, Germany) and reading of the plate at 405 nm.

Results

Selection of mAbs Identifying an HLA-A3–specific NK Receptor. In an attempt to identify NK cell clones specific for HLA-A molecules, we analyzed a large panel of clones derived from different individuals for their cytolytic activity against target cells transfected with different HLA class I al-

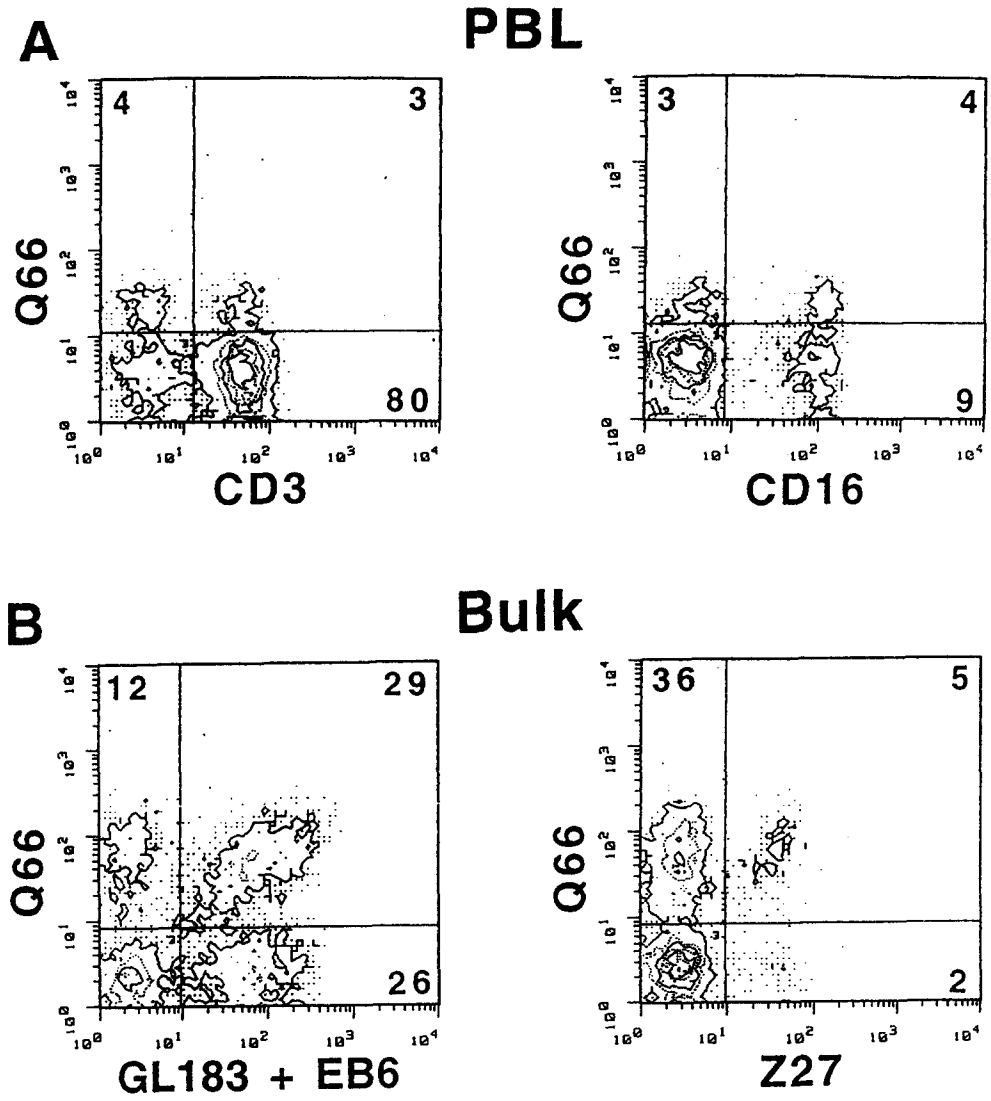
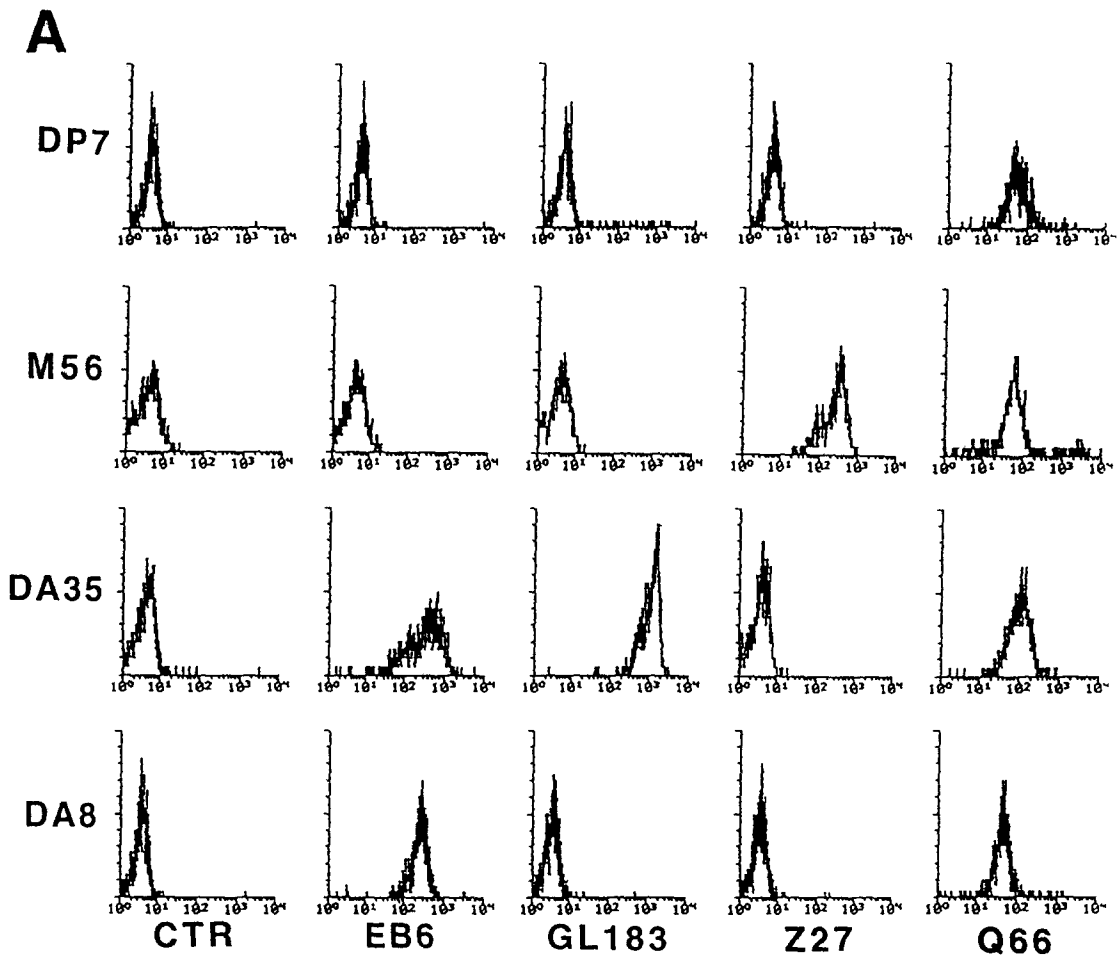


Figure 1. Cell distribution of Q66 mAb–reactive molecules. (*A*) Two-color cytofluorimetric analysis of the distribution of Q66-reactive molecules in PBL. Cells were stained with Q66 mAb followed by PE-conjugated goat anti–mouse IgM and with anti-CD3 (Leu4 mAb, IgG_1) or anti-CD16 (c127 mAb, IgG_1) followed by FITC-conjugated goat anti–mouse IgG_1. The contour plots were divided into quadrants representing unstained cells (*lower left*), cells with only red fluorescence (*upper left*), cells with red and green fluorescence (*upper right*), and cells with only green fluorescence (*lower right*). (*B*) Two color-cytofluorimetric analysis of the distribution of Q66 mAb–reactive molecules in a cultured $CD3^-$ 56^+16^+ bulk population. Cells were stained with Q66 mAb followed by PE-conjugated goat anti–mouse IgM and with a mixture of GL183 and EB6 (anti-p58.2 + anti-p58.1, both IgG_1) or Z27 (anti-p70, IgG_1) mAbs followed by FITC-conjugated goat anti–mouse IgG_1.

leles. To this end, C1R ($Cw4^+$) cells either untransfected or transfected with HLA-B7, -B27, -B51, -A1, -A2, -A3, or -A24 have been used as target cells. Other target cells included 221 cells transfected with HLA-Cw3 or -Cw4 (26). Among the HLA-A alleles analyzed, HLA-A3 appeared to exert the most marked protective effect. The majority of HLA-A3–specific clones, however, also recognized other HLA class I alleles and expressed the corresponding receptors. However, mAbs to these receptors, including EB6 (anti-p58.1) (8), GL183 (anti-p58.2) (7), Z27 (anti-p70) (9), and XA185 (anti-CD94) (22) did not restore lysis of C1R-A3 target cells. To facilitate the identification of the receptor molecule(s) involved in HLA-A3 recognition, we selected NK clones displaying specificity only for HLA-A3 molecules. Among these NK clones, the DP7 clone was further expanded and used for mouse immunization. The hybridoma supernatants were first screened for their ability to reconstitute lysis of C1R-A3 by the immunizing DP7 clone. Selected mAbs were further analyzed for their pattern of reactivity with DP7 clone, C1R-A3 target cells, as well as other NK cell clones specific or not for the HLA-A3 allele. mAbs Q66 (IgM) and Q241 (IgM) specifically reacted with the immunizing DP7 clone. In addition, they were found to selectively stain NK clones (derived from different individuals) displaying HLA-A3 specificity.

Analysis of the cell distribution of the surface molecules

Figure 2. (*A*) Expression of Q66 mAb–reactive molecules in NK cell clones. Four different $Q66^+$ clones were analyzed by indirect immunofluorescence for the expression of the NK receptors recognized by EB6 (p58.1), GL183 (p58.2), and Z27 mAbs (p70/NKB1). (*B*) Independent expression of Q66 mAb–reactive molecules and p58.1 receptors in a representative NK cell clone. DA8 clone ($EB6^+$, $GL183^-$, $Z27^-$, $Q66^+$) was further analyzed after mAb-mediated modulation of EB6 molecules (see Materials and Methods): Q66 mAb–reactive molecules do not comodulate with EB6 molecules. Dark profiles represent the mAb-reactive cells. White profiles represent cells stained with the isotype-specific anti–mouse antiserum.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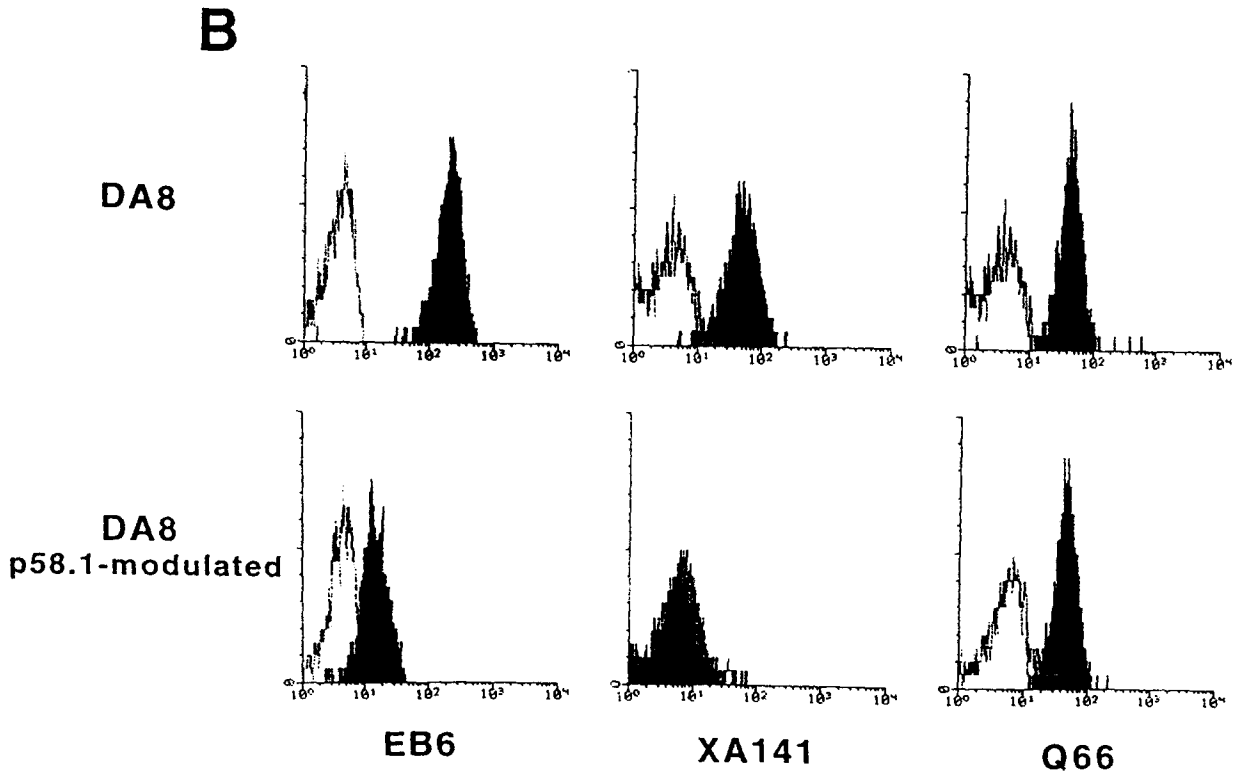
B
DA8
DA8
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recognized by Q66 and Q241 mAbs revealed an identical pattern of reactivity both in polyclonal NK cell populations and NK cell clones. Since Q66 mAb consistently gave a brighter staining than Q241 mAb, immunofluorescence studies were carried out primarily with this mAb. Fig. 1 *A* shows the pattern of reactivity of Q66 mAb with fresh PBL: it is evident that $Q66^+$ cells represent a subset of $CD16^+$ NK cells. In addition, a small but sizeable fraction of $CD3^+$ cells coexpressed Q66. This type of distribution is similar to that of previously described HLA class I–specific NK receptors, including p58.1, p58.2, p70/NKB1, and CD94 molecules (7–10, 23–25). Fig. 1 *B* shows a cultured polyclonal population enriched in $CD3^-16^+$ cells. $Q66^+$ cells stained only a fraction of the cell population analyzed; moreover, these cells could be distinguished from cells expressing p58 molecules (as revealed by staining with a mixture of EB6 and GL183 mAbs) or p70/NKB1 (as revealed by staining with Z27 mAb). Fig. 2 *A* shows four representative $CD3^-16^+$ $Q66^+$ NK cell clones. Clone DP7 (i.e., the immunizing clone) reacted with Q66 mAb, but not with mAbs specific for other NK receptors. Clone M56 coexpressed p70/NKB1, while clones DA8 and DA35 coexpressed p58.1 or both p58.1 and p58.2 receptors, respectively. In Fig. 2 *B* we further analyzed whether, in clone DA8, Q66 and p58.1 molecules were associated at the cell surface. The XA141 mAb (anti-p58.1, IgM) induced surface modulation of p58.1 molecules, as revealed by the sharp reduction in fluorescence intensity (using either EB6 or XA141 mAb). In contrast, the surface expression of Q66-reactive molecules was unchanged (20). These data indicate that the two molecules are not physically associated at the cell surface.

$Q66^+$ NK Clones Display Specificity for the HLA-A3 Allele. The following experiments have been designed to define whether the Q66 mAb–reactive molecules indeed represented the NK receptor for the HLA-A3 allele. In a first group of experiments, NK clones that did not recognize HLA-C or HLA-B alleles (and did not express p58 or p70 molecules) were analyzed for their reactivity with Q66 mAb. $Q66^+$ and $Q66^-$ clones that belong to this selected group were first analyzed for their ability to lyse four B-EBV cell lines, selected among 15 cell lines characterized by either homozygous or heterozygous HLA class I haplotypes. Selection was based on the expression of appropriate combination of HLA-A, -B, and -C alleles. It is evident (Fig. 3 *A*) that two cell lines (HOM2 and WT100BIS) were resistant to lysis by $Q66^+$ clones, while the other two (SP0010 and A51) were efficiently lysed. According to the HLA haplotype of the four B-EBV target cell lines (see Table 1), it is possible to conclude that neither HLA-C nor HLA-B alleles are involved in the protective effect. Indeed, the two protected cell lines expressed Cw1 or Cw4, respectively, belonging to the NK-defined HLA-C specificities, characterized by S77/N80 and N77/K80, respectively (4, 29); moreover, they expressed HLA-B27 or -B35, which belonged to the Bw4 or Bw6 supertypic specificities, respectively. The analysis of the expressed HLA-A alleles suggested that HLA-A3 and HLA-A11 conferred protection. The target cell lines that were susceptible to lysis expressed HLA-C and HLA-B alleles belonging to the same groups of the protected targets, but lacked HLA-A3 or -A11 (they were both homozygous for HLA-A2). These data suggest that the HLA-A3 and -A11, but not -A2, are recognized by $Q66^+$ clones. The $Q66^-$ clones lysed, with similar efficiency, HOM2 and SP0010 cell lines, expressing HLA-A3 and -A2, respectively (Fig. 3 *B*). To further document the specificity of $Q66^+$ clones, they were analyzed for the ability to lyse C1R cells transfected with different HLA-A alleles, including -A1, -A2, -A3, and -A24 (-A11 was not available). $Q66^+$ clones efficiently lysed C1R cells untransfected or transfected with HLA-A1, -A2 (Fig. 3 *C*), and -A24 (not shown). In contrast, the expression of HLA-A3 conferred protection from lysis (>50% inhibition of lysis). On the other hand, $Q66^-$ clones (Fig. 3 *D*) efficiently lysed all the above transfected target cells. Although not shown, C1R cells transfected with HLA-B7 or HLA-B2705 alleles or 221 cells transfected with Cw3 or Cw4 alleles were efficiently lysed by all $Q66^+$ clones used above (not shown). Thus, the cell distribution of Q66-reactive molecules, together with the pattern of cytolysis of $Q66^+$ versus $Q66^-$ NK clones, strongly suggested that Q66 mAb–reactive molecules represent the receptor for the HLA-A3 allele. We further analyzed whether the addition of Q66 mAb to the cytolytic assay could restore lysis of HLA-A3–protected targets by $Q66^+$ NK cell clones. Table 2 shows the cytolytic activity of a group of representative $Q66^+$ clones against $HLA\text{-}A3^+$ (C1R-A3 and HOM2) or $HLA\text{-}A11^+$ (WT100BIS) target cells (the $HLA\text{-}A2^+$ SP0010 target cells lacking A3 and A11 alleles were used as control). It is evident that addition of Q66 mAb resulted in target cell lysis. As a control, we used the anti–class I mAb A6-136 (IgM) previously shown to efficiently interfere with the interaction between HLA class I molecules and NK receptor (21) and the anti-CD56 mAb A6-220 (IgM). While the A6-136 mAb restored lysis of protected target cells (Table 2), A6-220 mAb had no effect (not shown). These data support the notion that Q66 mAb–defined molecules represent inhibitory NK receptors specific for HLA-A3 and -A11 alleles. To further investigate the HLA-A allelic specificity of $Q66^+$ clones, as well as the possible functional relationship with other HLA class I–specific NK receptors, we analyzed clones coexpressing Q66 and Z27 (Bw4-specific) mAb-reactive molecules. In Table 3, the representative clones A15 and A31 were tested against target cells expressing appropriate class I alleles. Thus, while lysis of C1R-A3 was reconstituted by the addition of the Q66 mAb alone, Z27 mAb alone was sufficient to reconstitute lysis of C1R-B51 and SP0010 B-EBV cell line. On the other hand, the B-EBV cell line HOM-2, expressing both HLA-A3 and HLA-B27 (Bw4), could be efficiently lysed only in the presence of both mAbs. These data indicate that the two receptors function independently.

Screening of a cDNA Library and Isolation of Genes Encoding NK Receptors Characterized by Three Extracellular Ig Domains. Since repeated attempts to immunoprecipitate Q66-reactive surface molecules have failed (possibly related

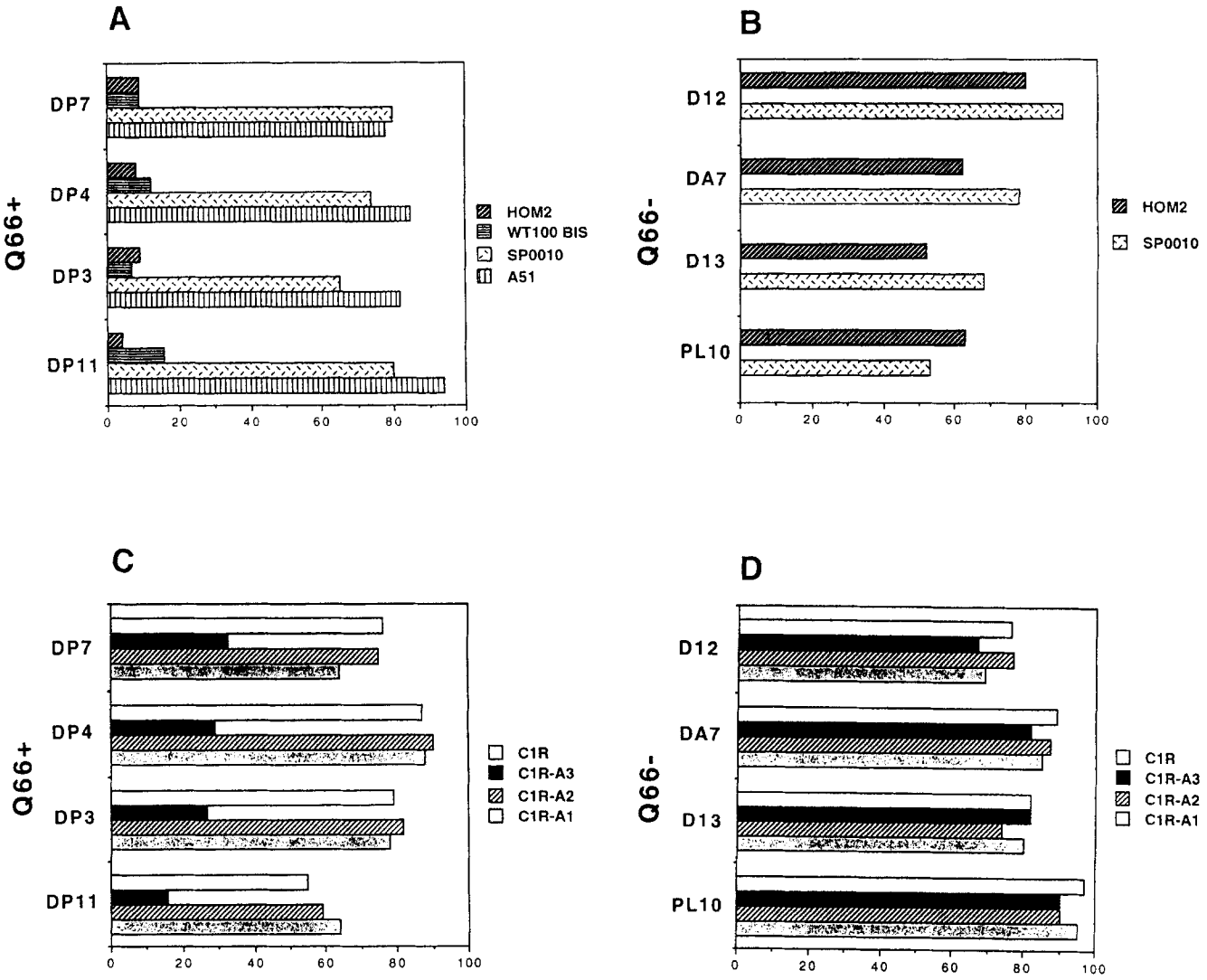

Figure 3. HLA-A3 expression on target cells confers protection from lysis by Q66$^+$ clones. (*A*) Four representative EB6$^-$, GL183$^-$, Z27$^-$, Q66$^+$ clones were assessed for cytolytic activity in a 4 h ^{51}Cr release assay against HOM2 (HLA-A3; -B27; -Cw1), WT100BIS (HLA-A11; -B35; -Cw4), SP0010 (HLA-A2; -B44; -Cw5), and A51 (HLA-A2; -B18,15; -Cw3,7) B-EBV cell lines. HOM2 and WT100BIS target cells were resistant to lysis, while SP0010 and A51 were efficiently lysed. The E/T ratio was 4:1. Data are expressed as a percent-specific ^{51}Cr release. (*B*) Four representative EB6$^-$, GL183$^-$, Z27$^-$, and Q66$^-$ clones were tested against HOM2 and SP0010. Both B-EBV cell lines were lysed. (*C*) The four Q66$^+$ clones shown in *A* were tested against C1R cells or C1R transfected with HLA-A3, -A2, or -A1. Although not shown, C1R-A24 cells were consistently lysed. (*D*) The four Q66-negative clones shown in *B* were tested against the same target cells of *C*.

to the IgM isotype of Q66 and Q241 mAbs), we attempted to identify these molecules by isolating the cDNA encoding for Q66 molecules in Q66$^+$ cell populations or clones. Since Q66-reactive molecules function as receptors for HLA class I molecules, we speculated that a possible candidate could be represented by a novel member of the p58 family (11). In an attempt to isolate these molecules, a 403-bp probe derived from the 5′ end of the 183 cl.6 sequence (nucleotides 88–491) was used to screen a cDNA library generated from the Q66$^+$ polyclonal CD3$^-$16$^+$ NK cell population derived from a healthy donor. Numerous cDNA clones were isolated: whereas most encoded for GL183/EB6–reactive molecules, two were found to encode for different molecules (cl.17.1 and cl.8.11). The hypothetical ORF of both cl.17.1 and cl.8.11 cDNAs encoded for molecules belonging to the Ig superfamily with three Ig-like domains. Although the cl.17.1 and cl.8.11 sequences were highly homologous, there was a single amino acid difference (I in pos.252 instead of T) and, more importantly, a 17 amino acid deletion in the extracellular portion proximal to the transmembrane region of cl.8.11-encoded protein. Both cDNAs lacked the first ATG initiation codon. Therefore, to obtain a complete coding sequence, we applied the 5′ RACE procedure, by using the same RNA used for preparation of the cDNA library. A full-length cDNA (cl.17.1C) that corresponded to cl.17.1 cDNA (Fig. 4) was obtained. The ORF of cl.17.1C cDNA encoded for a 455–amino acid type I transmembrane pro-

Table 2. *Q66 mAb Selectively Restores Lysis of Target Cells Expressing HLA-A3 or -A11 Alleles*

	Target cell											
	C1R-A3 Added mAb			HOM2 (A3,B27,Cw1) Added mAb			WT100BIS (A11,B35,Cw4) Added mAb			SP0010 (A2,B44,Cw5) Added mAb		
Clone	–	Q66	Anti-HLA* class I	–	Q66	Anti-HLA class I	–	Q66	Anti-HLA class I	–	Q66	Anti-HLA class I
DP7	33‡	64	70	9	84	80	9	79	75	80	88	94
DP4	29	54	91	8	67	89	12	53	72	74	80	80
DP3	27	54	72	9	64	74	7	49	60	65	72	88
DP11	16	54	60	4	41	52	16	66	81	80	85	90

*The anti-HLA class I mAb used was the A6-136 previously described (21).
‡The results are expressed as the precentage of specific lysis in cytolytic assays at an E/T ratio of 4:1.

tein belonging to the Ig superfamily, consisting of an extracellular region composed of 319 amino acids, characterized by three Ig-like domains, a 20–nonpolar amino acid transmembrane portion, and a 95–amino acid cytoplasmic tail. As already described for the inhibitory forms of HLA-C–specific receptors (p58 molecules), the cytoplasmic tail contained an ITAM-like sequence consisting of two YXXL motifs spaced by 26 amino acids (11–14). A comparison of the nucleotide sequence of cl.17.1C with other p58-related sequences (11–14) revealed a high degree of homology with NKAT4 and two other sequences encoding for receptors characterized by three extracellular Ig-like domains (NKB1, NKB1B).

Isolation of Full-length cDNAs Encoding for Molecules Characterized by Three Ig-like Domains Expressed in Q66$^+$ and Q66$^-$ NK Cell Clones. On the basis of the nucleotide sequence of the cl.17.1C (and of the other homologous sequences NKAT4, NKB1, and p58), two PCR primers were designed. The "Ig3 up" forward primer contained the ATG initiation codon, while the "Ig3 down" backward primer was designed in the 3′ untranslated region. When used in combination, they amplified 1.4-kb full-length cDNAs. By the use of these PCR primers, 1.4-kb fragments were amplified in various NK cell clones displaying different specificities for HLA class I alleles. Two different groups of 1.4-kb cDNAs were amplified. Sequences belonging to the first group (AMB11 and D97.10) were highly homologous to the NKB1 and NKAT3 sequences (12, 13). The NKB1 cDNA was recently shown to encode for a three Ig-like domain NK receptor specific for the HLA-Bw4 supertypic specificity. Although not shown, NIH3T3 cells transfected with AMB11 cDNA were brightly stained by Z27 mAb, thus formally proving that also AMB11 cDNA encodes for the Bw4-specific NK receptor. The second group of cDNAs (AMC5 and cl.1.1) were exclusively amplified from Q66$^+$ NK cell clones. A comparison of the amino acid sequences of AMC5, NKAT4, cl.1.1, cl.17.1C, AMB11, and NKB1 is shown in Fig. 4. The proteins encoded by the second group of cDNAs (AMC5 and cl.1.1) showed a high degree of

Table 3. *Independent Function of HLA-A3–and Bw4-specific receptors in Q66$^+$Z27$^+$ NK Cell Clones*

	Target Cell																			
	C1R-A3				C1R-B51 (Bw4)				HOM2 (A3,B27,Cw1)					SP0010 (A2,B44,Cw5)						
	Added mAb				Added mAb				Added mAb					Added mAb						
Clone	—	Q66	Z27	Anti-cl.I‡	—	Q66	Z27	Anti-cl.I	—	Q66	Z27	Z27+Q66	Anti-cl.I	—	Q66	Z27	Z27+Q66	Anti-cl.I		
A15	27*	55	34	53	8	16	57	68	2	7	7	46	46	5	12	61	71	72		
A31	40	72	43	84	8	6	98	96	2	2	14	59	75	4	0	74	80	88		

*The results are expressed as the percentage of specific lysis in cytolytic assays at an E/T ratio of 4:1.
‡The anti-LHA class I mAb used was the A6-136 mAb (21).

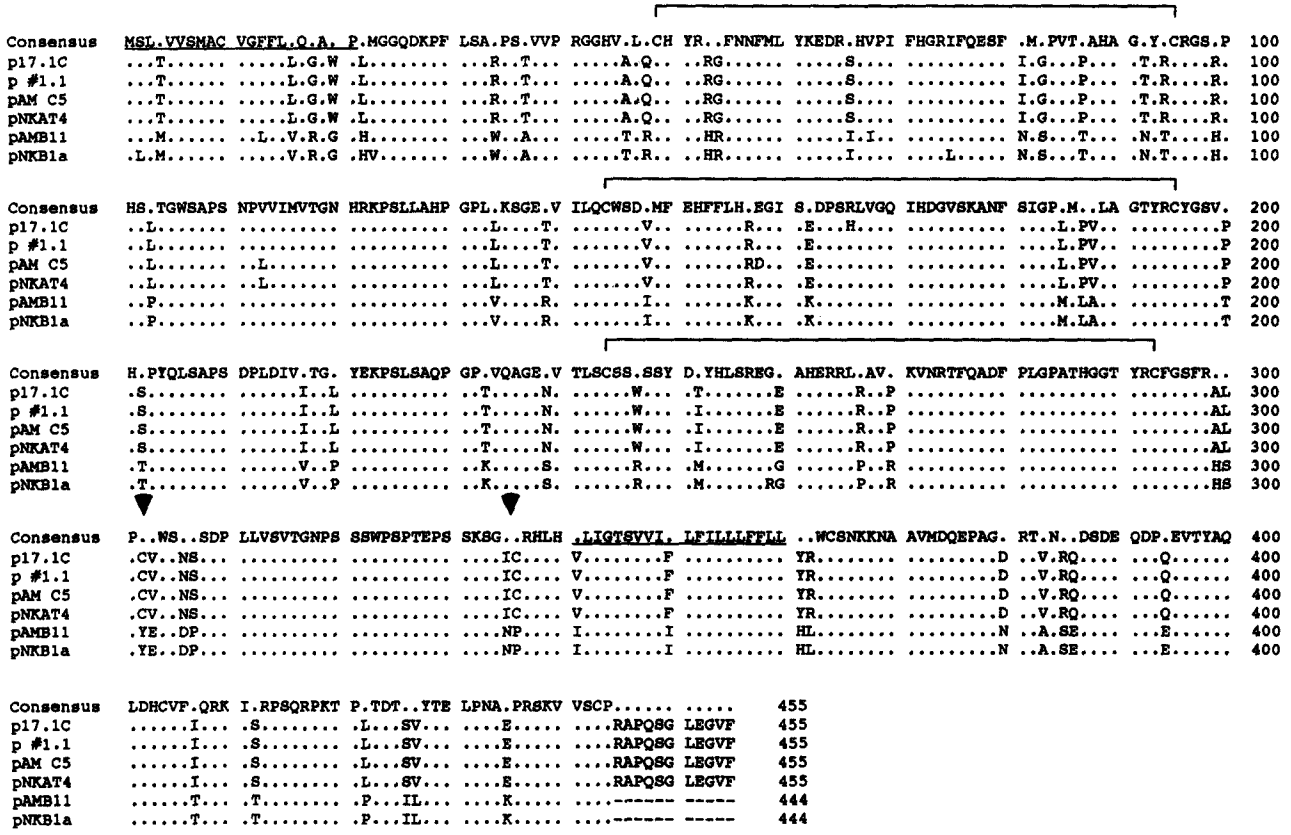

Figure 4. Alignment of the amino acid sequences coding for either Q66 and Q241 mAbs–reactive molecules or other 3 Ig domain NK receptors. The amino acid sequences of Q66 and Q241 mAbs–reactive molecules encoded by AMC5 and cl.1.1 cDNA clones are compared with sequences encoded by cl.17.1C, NKAT4, AMB11, and NKB1 cDNAs. Dashes were introduced to maximize homologies. Amino acids identical to the consensus are indicated by dots. The signal peptide and the transmembrane region in the consensus sequence are underlined; pairs of cysteines predicted to form Ig domains are connected by a line. Cysteines that are possibly involved in disulphide bridge formation are indicated by arrows. cDNA sequences have been submitted to EMBL with the following accession numbers: AMB11, X94262; 17.1C, X93595; 8.11C, X93596; cl.1.1, X94373; and AMC5, X94374.

homology with cl.17.1C protein, displaying only three amino acid differences. On the other hand, the protein sequences of AMC5 and cl.1.1 differed from the NKB1- and AMB11-encoded protein for ~50 amino acids in their extracellular portions. Moreover, both AMC5- and cl.1.1-encoded proteins displayed a cytoplasmic tail 11 amino acids longer than that of NKB1- and AMB11-encoded proteins (Fig. 4).

Correlation between the Surface Expression of Q66-reactive Molecules and the Presence of Defined Sequences Encoding for a Group of Three Ig-like Domains Receptors. To establish whether a correlation existed between the expression of Q66-reactive surface molecules and the presence of AMC5, cl.17.1C and cl.1.1 cDNA sequences (i.e., those exclusively amplified from $Q66^+$ clones), an appropriate oligonucleotide probe (probe Q) has been designed. This probe was specific for the nucleotide sequences of AMC5, cl.1.1, and cl.17.1C and was used to hybridize amplified PCR products from a large panel of $Q66^+$ and $Q66^-$ NK cell clones. Notably, these $Q66^+$ clones belong to the same group of those selected for the analysis of HLA class I allele specificity in cytolytic assay, i.e., those lacking other known NK receptors for HLA class I molecules (see above). As shown in Fig. 5, the probe Q hybridized with cDNA fragments amplified from $Q66^+$, but not from $Q66^-$ NK cell clones. Conversely, a probe specifically designed on the basis of the known cDNA sequences encoding for p70/NKB1 receptor (probe Z) did not hybridize with cDNA fragments amplified from $Z27^-$ $Q66^+$ clones. On the other hand, it did hybridize with cDNA from $Z27^+$ clones (not shown).

Transient Transfections in COS7 Cell Lines. COS7 cells were transfected with AMC5 or cl.1.1 cDNAs inserted in the pCR3 expression vector. After 48 h, cells were stained with Q66, Q241, Z27, EB6, and GL183 mAbs or with additional control mAbs of IgM isotype. COS7 cells transfected with AMC5 or cl.1.1 cDNAs were reproducibly stained by Q66 and Q241 mAbs, but not by the other mAbs (data not shown).

Generation of a Polyclonal Antiserum against Peptides Corresponding to Sequences Unique of AMC5/cl.1.1 and cl.17.1C. Based on the known amino acid sequences of NK receptors displaying three Ig-like domains, we synthetized a specific peptide corresponding to the COOH-terminal sequence of AMC5, cl.1.1, and cl.17.1 proteins (amino acids

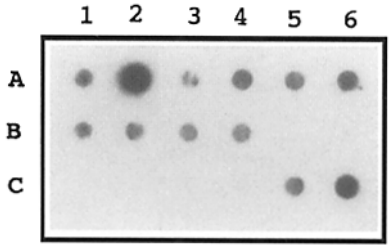

Figure 5. Amplification of specific cDNAs from $Q66^+$ or $Q66^-$ NK cell clones. The amplified fragments from $Q66^+$ $Z27^-$ (spotted in positions A1, A2, A3, A4, A5, A6, B1, B2, B3, and B4) and $Q66^-$ $Z27^+$ (spotted in positions B6, C1, and C2) NK clones were hybridized with the probe Q. These NK cell clones were derived from seven different donors. The AMB11 and D97.10 cDNAs (used as negative controls) were spotted in positions C3 and C4, while cl.1.1 and AMC5 cDNAs (positive controls) were spotted in positions C5 and C6, respectively.

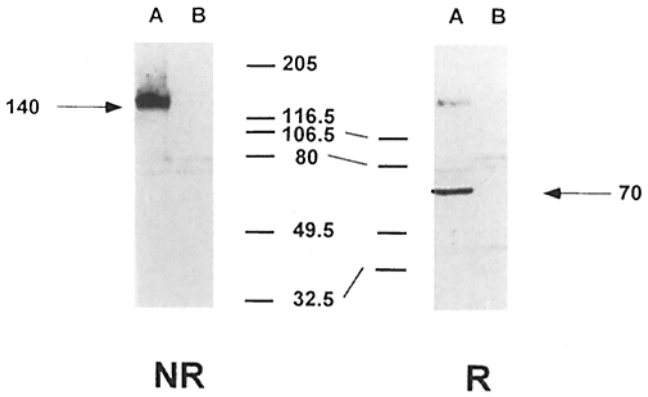

Figure 6. Western blot analysis of $Q66^+$ or $Q66^-$ NK cells. Western blot analysis of total cell lysates was performed in $Q66^+$ NK cell polyclonal populations (lanes *A*) and in the $Q66^-$ NK3.3 cell line (lanes *B*). SDS-PAGE was performed under reducing (R) or nonreducing (NR) conditions. Molecular mass markers (expressed in kilodaltons) are indicated. PGP3 polyclonal antiserum was used to stain the blot, followed by a chemiluminescence detection system.

441–455). Remarkably, NKB1 and NKAT3 protein sequences are characterized by a cytoplasmic tail shorter than that of AMC5, cl.1.1 and cl.17.1, which stops at amino acid 444. The 441–455 peptide was used to immunize a rabbit. The antiserum obtained (termed PGP3) was found to react with the immunizing peptide but not with irrelevant peptides (not shown). PGP3 was used to analyze, in Western blot, a panel of $Q66^+$ or $Q66^-$ NK cell lines; under nonreducing conditions, $Q66^+$ NK cells displayed 140-kD molecules that resolved in a single band of 70 kD under reducing conditions (molecules were therefore termed "p140"). No p140 molecules could be detected in $Q66^-$ NK cells (independent on the expression of p70/NKB1 receptors) (Fig. 6). Similar data were obtained by the analysis of $Q66^+$ polyclonal populations derived from two additional donors. These results strongly suggest that PGP3 specifically recognizes the proteins encoded by AMC5 and cl.1.1 cDNAs.

Biochemical Analysis of cl.1.1-encoded Molecules Expressed by NIH3T3 Cell Transfectants. To characterize the p140 molecules encoded by cl.1.1 cDNA, we analyzed the proteins expressed by the murine NIH3T3 cells transfected with the pCR3 cl.1.1 construct. As shown in Fig. 7, *upper panel*, only transfected cells were brightly stained by Q66 and Q241 mAbs, but not by other mAbs, including the p58- or p70-reactive EB6, GL183, or Z27 mAbs. In addition, the mean fluorescence detected in cell transfectants was similar to that of $Q66^+$ NK cell clones for both Q66 and Q241 mAbs. These data further confirm that both mAbs recognize the same molecule. No staining could be detected in NIH3T3 cells transfected with other constructs for p58 or p70 molecules (data not shown). NIH3T3 cells either untransfected or transfected with cl.1.1 were lysed and total cell lysates were analyzed by Western blot by using the PGP3 antiserum. As shown in Fig. 7, *lower panel*, under nonreducing conditions (*NR*), two bands of 140 and 70 kD were detectable in NIH3T3 cells transfected with cl.1.1 (*B*) and in a $Q66^+$ NK cell polyclonal population (*C*), but not in untransfected NIH3T3 cell line (*A*). Moreover, under reducing conditions (*R*), only a 70-kD band could be detected in the cl.1.1-transfected NIH3T3 cell line (*B*) and in the polyclonal $Q66^+$ NK cell population (*C*), see also Fig. 6. Thus, by comparison with the p70 receptor recognized by Z27 mAb, the molecular weight of the p140 molecules detected by PGP3 antiserum is consistent with a dimer of 3 Ig domain molecules. Since NIH3T3 cells transfected with cl.1.1 are brightly stained by Q66 and Q241 mAbs, but not by other mAbs specific for p58 or p70 NK receptors, these data strongly suggest that cl.1.1 encodes for Q66- and Q241-reactive molecules. Thus, data in both $Q66^+$ NK cell populations and in NIH3T3 cell transfectants are consistent with the expression of p140 molecules as disulphide-linked dimers. This finding is consistent with the presence, in the corresponding sequences, of two cysteins at positions 302 and 336, respectively (see Fig. 4).

Discussion

In the present study, we identified a novel surface molecule expressed by a subset of human NK cells that functions as receptor specific for defined HLA-A alleles. Molecular cloning revealed a high degree of homology with the p58/p70 family of NK receptors for HLA class I molecules (11–13). This receptor is characterized by three Ig-like extracellular domains, a transmembrane portion of 20 nonpolar residues and a 95–amino acid tail containing an ITAM-like motif. Remarkably, different from the previously identified p58/p70 receptors, it can be expressed as a disulphide-linked dimer of ~140 kD ("p140").

NK receptors specific for different groups of HLA-C and HLA-B alleles have recently been described and cloned. They function as inhibitory receptors which, upon binding their appropriate HLA class I ligand, transduce a negative signal to the NK cell, thus inhibiting target cell lysis (2, 11, 12). An activatory form of HLA-C–specific receptor has

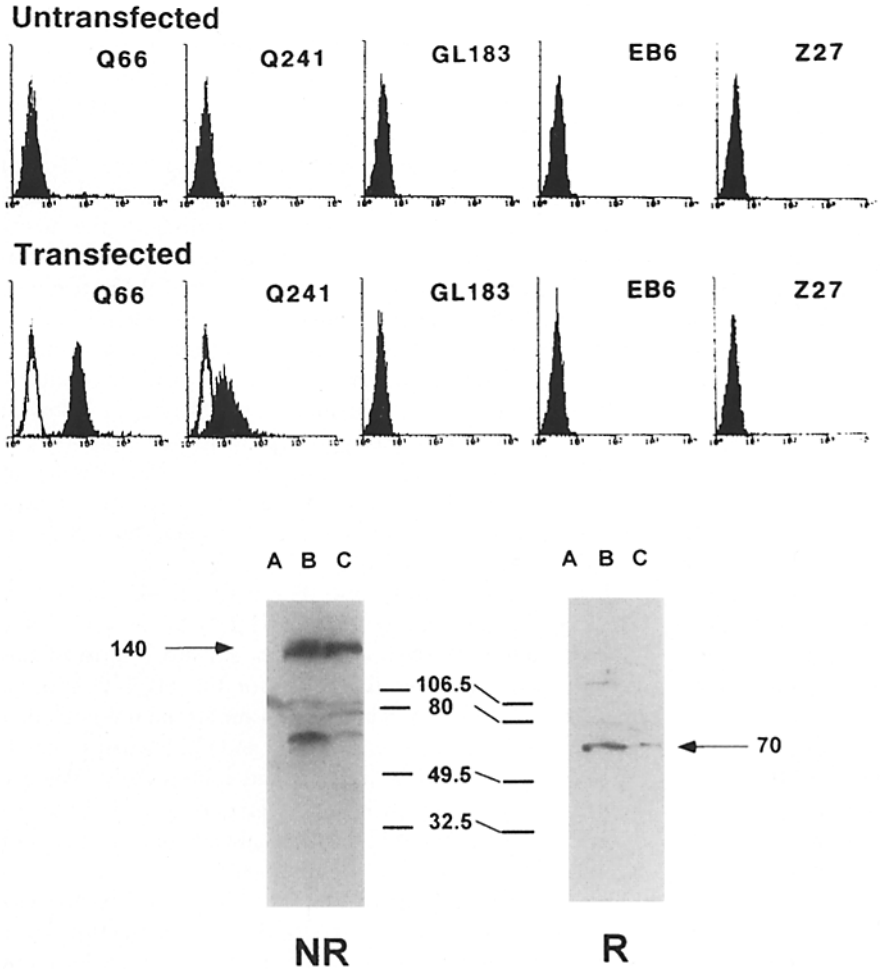

Figure 7. Cell-surface expression and Western blot analysis of Q66 and Q241 mAb–reactive proteins in cell transfectants. (*Top*) NIH3T3 cell line either untransfected or transfected with cl. 1.1. NIH3T3 cells were trypsinized, stained with Q66 or Q241 mAb, followed by a PE-conjugated goat antibody to mouse IgM or GL183, EB6, or Z27, followed by a goat antibody to mouse IgG, and analyzed by FACSort® (Becton Dickinson). Isotype-matched mouse Ig were used as controls. Dark profiles represent cells stained with the indicated mAb. White profiles represent controls stained with the second reagent only. (*Bottom*) Western blot analysis of total cell lysates of the NIH3T3 cell line untransfected (*A*), transfected with pCR3 cl. 1.1. (*B*), or of a $Q66^+$ NK cell polyclonal population (*C*). SDS-PAGE was performed under nonreducing (*left*) or reducing (*right*) conditions. Molecular mass markers (expressed in kilodaltons) are indicated. PGP3 polyclonal antiserum was used to stain the blot, followed by a chemiluminescence detection system.

recently been described (14, 30). However, this receptor is expressed only by certain individuals, and it differs from conventional inhibitory receptors in the transmembrane and cytoplasmic portions.

Litwin et al. (10) have previously described clones specific for different HLA-A alleles, including -A1, -A3, and -A69. Our present study provides the first molecular evidence for the existence of defined receptors for HLA-A alleles. Similar to the other HLA class I–specific receptors, the NK receptor for HLA-A3 is expressed by a subset of NK cells, functions as an inhibitory receptor, and may be coexpressed with other NK receptors at the single-cell level. Although present in all donors tested, its level of expression may be low. It should be noted that an unequivocal HLA-A3 specificity could be detected only in clones brightly stained by Q66 mAb. This finding is in line with data on other NK receptors, e.g., it has been shown that a critical number of p58.1 (EB6) molecules is needed to provide an efficient inhibitory signal to NK cells (5).

The specificity of p140 molecules for HLA-A3 or -A11 alleles has been clearly demonstrated by a number of experiments performed on C1R cells transfected with different HLA class I alleles or B-EBV cell lines expressing an informative HLA class I haplotype. It should be stressed that we selected $Q66^+$ NK cell clones displaying specificity only for HLA-A3 molecules. Consistently, these clones did not express p58 or p70/NKB1 molecules but did express CD94 molecules. In these clones, however, anti-CD94 mAb did not restore lysis of $HLA\text{-}A3^+$ target cells. These selected $Q66^+$ clones did not lyse cells expressing HLA-A3 or -A11, but lysed cells lacking these alleles. In all instances, target cells transfected with different HLA-C or -B alleles were efficiently lysed by $Q66^+$ clones. The specificity of p140 molecules for HLA-A3 or -A11 alleles was further substantiated by the ability of Q66 mAb to selectively restore lysis of $HLA\text{-}A3^+$ target cells by $Q66^+$ clones. Analysis of clones coexpressing p140 and p58 or p70/NKB1 receptors indicated that p140 molecules were not physically associated to any of these receptors. For example, mAb-induced modulation of p58.1 molecules did not affect the surface expression of p140 molecules. In addition, the receptors for Bw4 (p70/NKB1) and those for HLA-A3 (p140) were functionally independent as shown by the analysis of $Z27^+Q66^+$ clones. Thus, when tested against target cells coexpressing HLA-Bw4 and HLA-A3 alleles (see, for example, the cell line HOM-2), lysis was achieved only by

mAb-mediated masking of both receptors (Table 3). Similar results were obtained by the analysis of cell transfectants. Thus, Q66 mAb could restore lysis only when tested against C1R-A3, but not against C1R-B27. Restoration of lysis of C1R-B27 required the addition of Z27 mAb. A similar functional independency was observed in clones co-expressing p140 and p58 receptors (not shown). Taken together, these data indicate that p140 functions as an inhibitory receptor for HLA-A3 or -A11 alleles, but not for HLA-C or -B alleles. In addition, the finding that at least three different HLA-A alleles (-A1, -A2, and -A24) did not confer protection from $Q66^+$ clones clearly indicates that p140 receptor discriminates among HLA-A alleles. In this context, comparative analysis of the sequences of HLA-A alleles indicates that HLA-A3 and -A11 molecules display the highest homology in the amino acid sequences corresponding to the α1 domain (i.e., a region known to be involved in the NK-mediated recognition of HLA-C or -B alleles). Remarkably, -A3 and -A11 alleles did not differ from the -A2 allele in the amino acid positions 77 and 80, despite the different ability to provide target cell protection from $Q66^+$ clones. Thus, the amino acid positions 77 and 80 are not crucial for the NK-mediated recognition, as it occurs for HLA-C alleles.

Analysis of the molecular structure of the p140 molecule revealed a new member of the p58/p70 family of NK receptors displaying three extracellular Ig-like domains. However, this receptor is expressed as a disulphide-linked dimer of 140 kD. This suggests that recognition of HLA-A3 molecules may be mediated by the receptor in its dimeric form. Analysis of the amino acid sequences deduced from the cDNA nucleotide sequences showed two cysteines in the extracellular portion of p140 molecules proximal to the transmembrane region, thus differing from the other p58/p70 members. These cysteines may explain the expression of p140 as a disulphide-linked dimer. Notably, the p140 form is the prevalent or the exclusive form detectable in $Q66^+$ polyclonal NK cell population, while both p140 and p70 are well represented in NIH3T3 cells transfected with cDNA cl.1.1. This could reflect a less efficient assembly of monomers in cell transfectants. Interestingly, the ORF of cl.8.11C and cl.17.1C differed by a deletion of 17 amino acids in the extracellular portion proximal to the transmembrane region of cl.8.11C. A similar deletion has been described by D'Andrea et al. in the NKB1/NKB1B molecules (12). The mechanism involved in this deletion occurring in three Ig-like domains receptors remains to be defined, although a possible explanation could be a mechanism involving an alternative splicing. The isolation of different cDNAs for highly homologous proteins, all reactive with Q66 and Q241 mAbs, is compatible with the existence of allelic forms of the receptors. Notably, the existence of multiple homologous forms of receptors has been described also for p58 and p70/NKB1 (31).

In the present study, we describe only the inhibitory form of the receptor specific for HLA-A3 or -A11. We cannot exclude the existence of an activatory form of this receptor, as was recently shown for the HLA-C–specific receptors. We did not address this issue in the present study because of the IgM isotype of Q66 and Q241 mAbs, which could not be tested in a redirected killing assay. We are presently investigating whether sequences typical of the activatory forms of the NK receptors are present in a panel of $Q66^+$ clones from different individuals.

Interestingly, similar to p58 or p70, the HLA-A3–specific receptors are also expressed in a small fraction of T cells. It is likely that they could exert an inhibitory function on TCR-mediated T cell activation, as previously documented for both p58 or p70 (23–25).

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