Cloning and Functional Characteristics of Murine Large Granular Lymphocyte-1: A Member of the Ly-49 Gene Family (Ly-49G2)

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Summary

Large granular lymphocyte (LGL) 1 is a cell surface glycoprotein expressed on a subset (50%) of C57BL/6 natural killer (NK) cells. Immunoprecipitation experiments reveal that the LGL-1 protein exists as a disulfide-linked 40-kD homodimer. Functional studies of LGL-1$^+$ cells indicate that selected H-2^d target cells are not lysed efficiently by these interleukin (IL)-2–cultured NK cells. These findings suggested that LGL-1 may be a member of the Ly-49 gene family. Here we report the molecular cloning of the LGL-1 cDNA from a severe combined immunodeficient–adherent lymphokine-activated killer cell library transfected into Cos-7 cells and find LGL-1 to be homologous to the Ly-49 gene at both the nucleotide (85%) and amino acid levels (73%). Sequencing of our LGL-1 cDNA has revealed it to be nearly identical to the Ly-49G2 cDNA recently isolated by cross-hybridization with an Ly-49 probe. LGL-1 represents a type II transmembrane protein of 267 amino acids with its carboxyl end exposed extracellularly. The LGL-1 protein contains 11 highly conserved cysteine residues and a 25–amino acid transmembrane region. Southern blot analysis demonstrates that there are a number of homologous genes in mouse DNA that hybridize strongly to LGL-1. Northern analyses using poly A$^+$ RNA from LGL-1$^+$ NK cells indicate that LGL-1 is expressed as a 1.4 kb mRNA. Two-color flow cytometry analysis (FCA) of C57BL/6 splenic NK cells demonstrates that LGL-1 and Ly-49 label overlapping subsets of cells. FCA identifies four subsets of NK cells as defined by LGL-1 versus Ly-49 staining. We have sorted these individual subsets, expanded them in IL-2, and performed cytotoxicity experiments to determine their target cell profiles in relation to class I expression. Results of these studies are complex, but indicate that Ly-49 may not be the only molecule that recognizes class I as an inhibitory signal for cytotoxicity. LGL-1$^+$ cells also fail to lyse several H-2^d–expressing tumor targets and concanavalin A lymphoblasts from BALB/c but not C57BL/6 mice. This inhibition of lysis by LGL-1$^+$ NK cells is negated by addition of monoclonal antibody (mAb) 4D11 that recognizes the LGL-1 protein. When mAbs to the class I molecules H-2D^d and H-2L^d ($\alpha_1\alpha_2$ domains only) are added to cytotoxicity assays, LGL-1$^+$ cells lyse H-2^d targets very effectively. Therefore, LGL-1 recognizes regions of the class I–specific molecules H-2D^d and H-2L^d. This specificity distinguishes LGL-1 from Ly-49, whose killing was only reversed by antibodies to H-2D^d. The differential specificities recognized by LGL-1 versus Ly-49 support the hypothesis that this family of genes recognizes diverse class I molecules and regulates the lytic activity of NK cells.

NK cells have been defined for more than 20 years as a population of non-T cell, non-B cell, LGL capable of lysing a wide array of virally infected and tumor target cells in a non–MHC-restricted manner. Evidence that suggests class I MHC may indeed affect the ability of NK cells to lyse target cells has been accumulating. However, selected MHC class

I molecules appear to inhibit rather than enhance the lytic action of these cells. Data exists both for (1–9) and against (10–13) the ability of class I MHC molecules on target cells to effect recognition and lysis by NK cells. A number of recent studies using transfection of specific class I genes into target cells presents strong evidence suggesting their role as negative regulators of NK lysis (3, 5–7). Both in the human and murine systems, NK cells have been shown to be incapable of lysing target cells expressing high levels of class I molecules. In the murine system, Ly-49 has been shown to inhibit NK reactivity against target cells expressing H-2D^d and H-2D^k, and this specificity appears to reside at the $\alpha_1\alpha_2$ domain of H-2D^d (4). Ly-49 is expressed on a small subset (20%) of NK cells in B6 mice, but is polymorphic in its distribution on other strains of mice. Cloning and sequencing of the Ly-49 gene places it in the C-type lectin family of type II transmembrane proteins (14) that maps to chromosome 6 (15) along with other members of the NK complex (16).

This laboratory has previously generated and characterized an mAb (4D11) that recognizes a nonallelic determinant, referred to as LGL-1, that is present on 50% of murine NK cells in B6 mice (17). We also have demonstrated that LGL-1$^+$ cells, upon IL-2 activation and culture, are not capable of lysing selected tumor cell targets expressing high levels of H-2^d such as P815 and L5178Y (18). LGL-1 has been biochemically characterized as a 40-kD disulfide-linked homodimer. Therefore, the functional and biochemical characteristics of the LGL-1 molecule are quite similar to those of the Ly-49 molecule. In this report, we present data on the cloning and sequencing of the LGL-1 cDNA along with the functional properties of NK cells expressing LGL-1 and Ly-49. Sequencing data have revealed that LGL-1 is a member of the Ly-49 gene family and has a high degree of homology at both the nucleotide (85%) and amino acid (73%) levels. We also have performed cell sorting of NK cells expressing LGL-1 versus Ly-49, and we have analyzed the functional properties of the four resulting subsets after culturing these cells in IL-2. Analysis of both tumor targets and Con A blasts indicate that both LGL-1$^+$ and Ly-49$^+$ NK cells are not able to lyse target cells expressing high levels of H-2^d. Ly-49$^+$ NK cells appear to be inhibited even when target cells express low levels of class I on their surfaces. The presence of multiple Ly-49–related gene products on NK cell subsets implies a complicated mechanism of NK lytic function regulation.

Materials and Methods

Cloning of LGL-1. Poly A$^+$ RNA obtained from CB.17 severe combined immunodeficiency–adherent lymphokine-activated killing (SCID-ALAK)[1] NK cells was used to prepare a cDNA library and were inserted into the pME18S plasmid expression vector (Stoneman,

E., P. A. Mathews, V. Kumar, and M. Bennett, manuscript submitted for publication). Cos-7 cells were transfected using a modification of the Seed-Aruffo method (19). Cells were grown to 70% confluency in DMEM, washed, and 10 μg of plasmid DNA was added in a 1:50 dilution of Lipofectamine (GIBCO BRL, Gaithersburg, MD). After 5 h at 37°C, the cells were washed once in DMEM and trypsinized to remove adherent cells. After washing once in DMEM + 10% fetal bovine serum (FBS), the cells were replated and grown for 48–72 h at 37°C. Cells were detached and collected with HBSS (Ca^{2+} and Mg^{2+} free) + 0.5 mM EDTA + 0.02% azide after incubation for 30 min at 37°C. Cells were washed and stained with a 1:500 dilution of mAb 4D11 and incubated on ice for 1 h. Panning was performed for 2–3 h at room temperature using an F(ab')$_2$ goat anti-rat Fc-specific antibody (10 μg/ml; Jackson Laboratories, Bar Harbor, ME) attached to petri plates (Falcon 1007). Nonadherent cells were gently removed by washing three times with HBSS + EDTA + 0.02% azide + 5% FBS. Adherent 4D11$^+$ Cos-7 cells were disrupted with 0.4 ml of a 0.6% SDS solution containing 10 mM EDTA for 5–10 min at room temperature. The solution was adjusted to 1 M NaCl and placed on ice overnight. After centrifugation, plasmid DNA was extracted from the supernate with phenol/phenol–chloroform/chloroform, precipitated, and resuspended in 20 μl Tris-EDTA buffer and used to transform DH5α cells (GIBCO BRL, Gaithersburg, MD). Bacterial cells were grown in Luria-Bertani broth (LB) + 50 μg/ml ampicillin overnight, and plasmid DNA was extracted and used for successive rounds of Cos-7 transfection and panning. After the third round of transfection, the bulk population of Cos-7 cells was stained with 4D11 and FITC goat anti-rat γ2a and examined by flow cytometry analysis (FCA) for 4D11$^+$ cells. After the fourth round of transfection (resulting in ∼10% 4D11$^+$ cells), plasmid isolation, and DH5α transformation, 50 colonies were isolated. Miniplasmid preps were made from each and pooled into five groups of 10, transfected into Cos-7 cells, and 4D11$^+$ cells were determined by FCA. In this manner, a single LGL-1$^+$ clone was isolated that, upon transfection and expression in Cos-7 cells, resulted in ∼40% 4D11$^+$ cells.

DNA Sequencing. Plasmid cDNA containing the LGL-1 insert was cleaved with EcoRI and NotI, digested with HaeIII, AluI, or Sau3A, and cloned into the appropriate restriction site of M13mp18. Single-stranded template DNA was purified and sequenced with Sequenase (United States Biochemical Corp., Cleveland, OH). Both strands of all possible fragments were sequenced as previously described (20).

Southern Blotting. DNA for Southern blotting was prepared from mouse and rat spleens and digested with HindIII, PvuII, SstI, or EcoRI. Electrophoresis and blotting were performed as described previously (20). Hybridization was performed with the 1.0-kb insert from the EcoRI–NotI–digested LGL-1 plasmid DNA. The LGL-1 probe was synthesized by the random-primed method (GIBCO BRL) using [α-^{32}P]dATP as the label. Low stringency conditions consisted of hybridization at 42°C in formamide followed by washes at 50°C in 0.2 × SSC + 0.1% SDS. Higher stringency conditions included washes at 65°C in the same wash buffer.

Northern Blotting. LGL-1$^+$ and LGL-1$^-$ subsets were isolated from splenic B6 NK cells by cell sorting and were expanded in culture with IL-2. Poly A$^+$ RNA was extracted (Microprep; Pharmacia Fine Chemicals, Piscataway, NJ), electrophoresed on a 1% agarose/formaldehyde gel, and blotted onto a nylon membrane (Amersham Corp., Arlington Heights, IL) in 10 × SSC. Blots were hybridized using an α-^{32}P-labeled LGL-1 probe at 10^6–10^7 cpm/ml in 5 × SSC + 0.1% SDS at 65°C for 24–72 h, washed

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[1] *Abbreviations used in this paper:* FBS, fetal bovine serum; FCA, flow cytometry analysis; LB, Luria-Bertani broth; RADCC, reverse antibody-dependent cellular cytoxicity; SCID-ALAK, severe combined immunodeficiency–adherent lymphokine-activated killing.

in 0.2 × SSC + 0.1% SDS at 65°C for 15 min, and exposed to film for 24–72 h.

Immunoprecipitation and SDS-PAGE. Cos-7 and NK cells were radiolabeled with ^{125}I using the lactoperoxidase method and immunoprecipitated as previously described (21). Briefly, 5–10 × 10^6 cells were radiolabeled, lysed in 0.5% Triton X-100, and solubilized membranes immunoprecipitated with mAb 4D11 or a control rat IgG2a cross-linked to protein G–Sepharose (Pharmacia, Uppsala, Sweden). After extensive washing, immune complexes were eluted in reducing and nonreducing buffers and electrophoresed on a 10% SDS-PAGE. Gels were dried and exposed to film for various times.

Antibodies. All mAbs used were derived from 2× salt-cut ascite preparations. Rat IgG2a mAbs included 4D11 (LGL-1) and a control Rγ2A. Mouse IgG2a mAbs included PK136 (NK-1.1), A1 (Ly-49), and (Ly-2.2). Mar18.5 (goat anti–mouse K) was used as a facilitating antibody along with MT4 to eliminate CD4$^+$ cells during NK enrichment. mAb 2.4G2 is a rat IgG that specifically reacts with the FcγRII/III on the surface of murine NK cells and was used to stain target cells. The following antibodies were obtained from PharMingen (San Diego, CA) to either stain tumor targets for class I expression by FCA or as blocking antibodies in cytotoxicity assays: α-H2-K^b (clone AF6-8815), α-H2-K^d (clone SF1-1.1), α-H2-D^b (clone KH95), α-H2-D^d (clone 34.2.12), and α-H2-D^k (clone 15.5.5). Antibodies to specific domains were kindly provided by Dr. David Margulies (National Institute of Allergies and Infectious Diseases, Bethesda, MD) and included α-H2-D^{d}α$_1$α$_2$ (clone 34.5.8S), α-H2-L^{d}α$_1$α$_2$ (clone 30.5.7S), α-H2-D^{d}α$_3$ (34.2.12), and α-H2-L^{d}α$_3$ (28.14.8S) (22, 23, 23a).

Target Cells and Cytotoxicity Assays. All tumor cell targets were propagated in RPMI + 5% FBS as previously described (18). Con A lymphoblasts were prepared from splenocytes of C57BL/6, BALB/c, or B6 × BALB/cF1 as described by Chadwick and Miller (24). Essentially, single-cell suspensions were prepared at 10^6 cells/ml in RPMI + 10% FBS, including 3 μg/ml of Con A in T-25 flasks. Cells were incubated for 40–48 h at 37°C. Lymphoblasts were isolated from nonviable cells by separation on Lympholyte-M (Accurate Chemical and Science Corp., Westbury, NY). Cells were washed once in RPMI and prepared for radiolabeling with ^{51}Cr. ^{51}Cr release assays were performed as previously described (21). Cytotoxicity assays using mAb involved the inclusion of the antibodies for the duration of the 4-h assay at a final concentration of 2 μg per well. All class I antibodies used in cytotoxicity assays were added directly to the assay at a concentration of 1 μg/well. Spontaneous release of Con A blasts was <25% and tumor targets <20% in all assays.

FCA. FCA was performed on a FACScan® (Becton Dickinson & Co., Mountain View, CA). NK cells were stained essentially as previously described (21). mAb 4D11 was directly labeled either with PE or FITC. mAb A1 was used as a 2× salt-cut preparation at a 1:1,000 dilution followed by a FITC αMouseIgG2a (CALTAG Laboratories, Inc., South San Francisco, CA).

NK Cell Isolation and Cell Sorting. NK cells from B6 mice were enriched from spleens of 12–16-wk-old animals by collecting nylon wool nonadherent cells, depletion of T cells using mAbs to Ly-2.2 and CD4, and removal of B cells by immunoabsorbance to plates as described previously (18). Routinely, 75–80% of the resulting cells were NK1.1$^+$. Cells were stained as described above with mAb A1 followed by a combination of FITC αMouseIgG2a plus 4D11 PE. Cells were sorted on either a FACStar® (Becton Dickinson & Co.) or an Epics 753 (Coulter Electronics, Hialeah, FL) cell sorter. NK cell subsets were isolated and cultured in high dose IL-2 (10^3 U/ml) for 6–8 d as described previously (18).

Results

Cloning of LGL-1 cDNA. A modification of the Seed and Aruffo (19) method for transfection, enrichment, and isolation of cDNAs that encode cell surface proteins has been used to clone LGL-1 cDNA. An NK-cDNA library derived from CB.17 SCID-ALAK cells that had been cloned into the pME18S expression vector was obtained. Lipofectamine was used as the method of transfecting Cos-7 cells to obtain maximal efficiency, followed by panning LGL-1$^+$ cells with mAb 4D11 and transformation of DH5α cells with enriched plasmid preparations. Plasmid cDNA from the fourth round of enrichment was used to transform DH5α cells that were plated onto LB + ampicillin plates. 50 colonies were selected, and plasmid preparations were pooled, transfected into Cos-7 cells, and examined for LGL-1 expression by FCA. A single clone (No. 16) containing the LGL-1 cDNA was isolated (data not shown).

Immunoprecipitation of LGL-1 from Transfected Cos-7 Cells. To confirm that clone No. 16 contained cDNA encoding the LGL-1 protein, immunoprecipitation of Cos-7 cells transfected with clone No. 16 plasmid cDNA was performed. Fig. 1, A and B, demonstrates the results of immunoprecipitation followed by SDS-PAGE under both nonreducing (Fig. 1 A) and reducing conditions (Fig. 1 B). Fig. 1 A demonstrates that plasmid cDNA from clone No. 16 encodes an ∼87-kD protein under nonreducing conditions and a homodimer of ∼40 kD under reducing conditions (Fig. 1
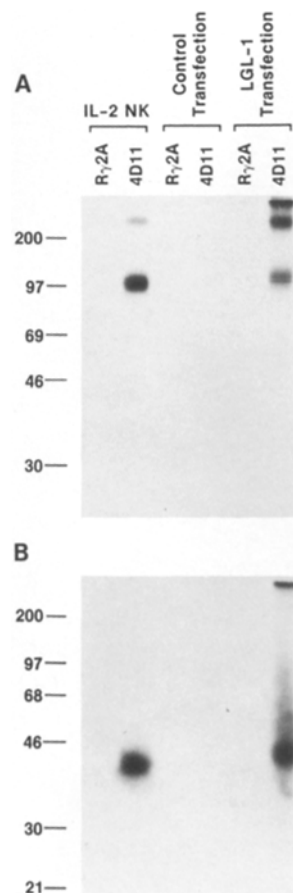

Figure 1. Immunoprecipitation of Cos-7 cells transfected with the cloned LGL-1 plasmid. (A) Cos-7 cells were either transfected with a control plasmid DNA preparation (lanes 3 and 4) or plasmid DNA from LGL-1 (lanes 5 and 6). IL-2–cultured NK cells were included for comparison in lanes 1 and 2. All cells were labeled with ^{125}I and immunoprecipitation performed with either an isotope control mAb (Rγ2A) (lanes 1, 3, and 5) or mAb 4D11 (lanes 2, 4, and 6). 10% SDS-polyacrylamide gels were electrophoresed under nonreducing conditions. (B) Identical 10% SDS-polyacrylamide gels electrophoresed under reducing conditions.

B), consistent with that previously reported for the LGL-1 protein. Lanes *1* (control) and *2* (4D11) represent immunoprecipitates from IL-2–cultured B6 NK cells; lanes *3* (control) and *4* (4D11) represent negative control–transfected cell lysates; and lanes *5* (control) and *6* (4D11) represent cells transfected with plasmid cDNA from clone No. 16. It appears from these gels that transfection of Cos-7 cells with LGL-1 cDNA generates a protein that is of a slightly higher molecular weight than that seen in NK cells. Both the nonreduced 87-kD and reduced 40-kD bands migrate somewhat slower when immunoprecipitated from transfected Cos-7 cells than from NK cells, probably because of differences in glycosylation of the LGL-1 protein in Cos-7 cells. However, the molecular weight of the proteins immunoprecipitated with 4D11 from transfected Cos-7 cells was consistent with that obtained from NK cells, demonstrating that clone No. 16 encodes the LGL-1 protein.

Sequencing of LGL-1 cDNA. Plasmid encoding the LGL-1 protein was digested with EcoRI and Not1, and was found to contain a 1.0-kb cDNA fragment. Sequencing of this fragment revealed 1,042 bp encoding a protein of 267 amino acids (Fig. 2 *A*). An untranslated 30-bp segment resides at the 5' region and a 210-bp segment at the 3' region of the LGL-1 cDNA. Comparison of LGL-1 to the reported Ly-49 cDNA sequence reveals a high degree of homology at both the nucleotide and amino acid levels. Fig. 2 *B* demonstrates a best-fit alignment of the three most recently characterized members

of the Ly-49 gene family. In this figure, the amino acid sequence of LGL-1 is compared with that of Ly-49 and SW5E6, a recently cloned molecule (24a) defining a subset of NK cells that may play a role in regulating hematopoiesis (Yu, Y. Y., J. Roland, V. Kumar, and M. Bennett, manuscript submitted for publication). SW5E6 also has been determined to be an Ly-49 family member with ~65% homology to Ly-49 at the protein level. The biological role of 5E6^{+} NK cells pertaining to class I inhibition is currently under investigation. LGL-1 and Ly-49 display an overall homology of 85% at the nucleotide level with a corresponding 73% amino acid homology. The amino acid composition of this type II transmembrane protein indicates that it belongs in the Ly-49 gene family of proteins. A presumed intracellular domain of 44 amino acids is found at the NH2 terminus of the LGL-1 protein that contains possible phosphorylation sites for casein kinase, protein kinase C, cAMP–dependent protein kinase, and tyrosine kinases. This is followed by a 25–amino acid transmembrane region. The extracellular domain of the LGL-1 protein contains 10 cysteine molecules that are highly conserved in the Ly-49 gene family. These highly conserved cysteine molecules would appear to account for the strong disulfide-linked nature of LGL-1, Ly-49, and the SW5E6 homodimers. The LGL-1 cDNA also contains three N-linked glycosylation motifs that account for the glycosylated nature of the LGL-1 protein (21). A unique feature of the LGL-1 protein is a four–amino acid segment (RPGN) that is not
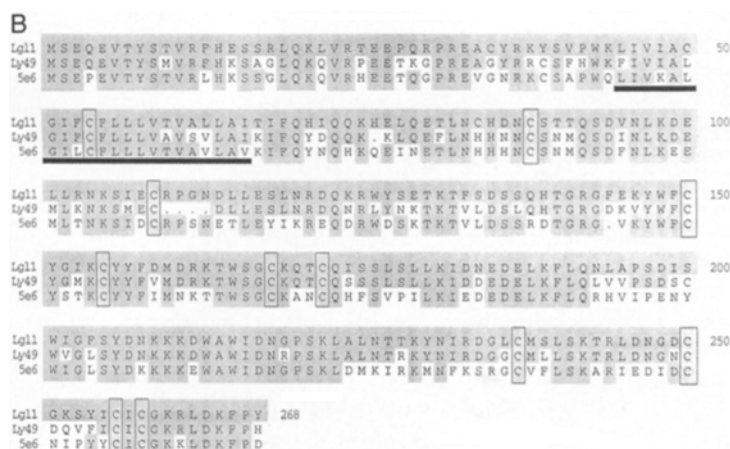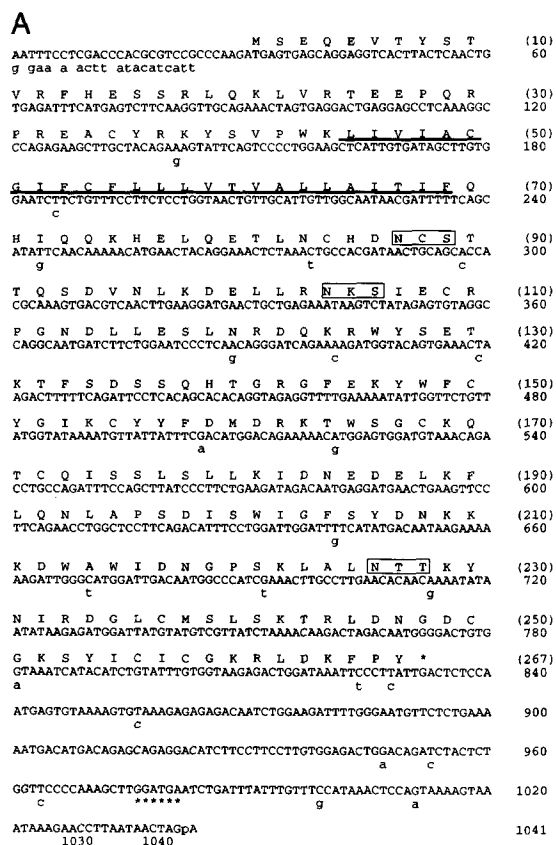

Figure 2. Nucleotide sequence and proposed amino acid sequence of LGL-1 compared with Ly-49 and 5E6. (*A*) The complete nucleotide sequence of the 1.0-kb fragment of cDNA encoding the LGL-1 molecule is presented. The nucleotide number is shown to the right of each line, and the amino acid number is shown in parentheses. 30 bp at the 5' and 210 bp at the 3' end of the molecule are untranslated. Differences in nucleotide sequence between LGL-1 and Ly-49G2 are notated as lower case letters directly beneath the LGL-1 sequence. The 801-bp coding region translates into a 267–amino acid protein as noted above the nucleotide sequence. The proposed transmembrane region is underlined and the three potential N-linked glycosylation sites are boxed. (*B*) Alignment of the proposed amino acids of LGL-1 compared with Ly-49 and SW5E6. The transmembrane region of the protein is underlined. Shaded areas represent areas of homology as opposed to unshaded areas, which represent differences in amino acid content. Homologous cysteines are boxed, and dots represent deletions of specific amino acids.

present in the Ly-49 protein. Analysis of the protein sequence of LGL-1 indicates that it belongs in the family of type II integral membrane proteins, along with Ly-49, with which it shares the highest degree of homology.

Northern Blot Analysis. To determine if LGL-1 mRNA correlates with NK cells expressing the LGL-1 protein, as well as to determine the size of the mRNA transcript, Northern blotting was performed. NK cells from B6 mice were sorted into LGL-1$^+$ and LGL-1$^-$ populations, expanded in IL-2, and used to isolate poly A$^+$ RNA. A 1.4-kb message was demonstrated in LGL-1$^+$ cells (Fig. 3, *top*); however, LGL-1$^-$ cells did not display any significant hybridization, demonstrating that the LGL-1 message is confined to NK cells expressing the LGL-1 protein. A control probe for β-actin was later applied to the same blot (Fig. 3, *bottom*) and indicated that there was approximately the same amount of mRNA present in each lane. The observed hybridization was not caused by crosshybridization between the LGL-1 cDNA probe and Ly-49 since Ly-49$^+$ cells are found in both LGL-1$^+$ and LGL-1$^-$ populations at the same levels (∼25%). These results indicate that although there appear to be a number of Ly-49 gene family members, Northern blot analysis under stringent conditions allows for the specific detection of individual family members.

Southern Blot Analysis. Restriction enzyme digests of rat and CB.17 SCID mouse DNA, followed by blotting and hybridization with our 1.0-kb LGL-1 cDNA probe, reveal a large number of hybridizing bands (Fig. 4 *A*). In this figure, hybridization was performed at 42°C, followed by washes at 50°C in 0.2 × SSC plus 0.1% SDS (low stringency conditions). These results demonstrate that as many as 11 bands in the rat and up to 9 bands in the mouse hybridize with the LGL-1 cDNA. However, after washing the blot at much higher stringency (65°C), virtually all of the rat bands and several of the mouse bands are no longer present, indicating only weak homology of these bands (Fig. 4 *B*). Data with human DNA did not reveal any specific hybridization after low or high stringency washes. Our data using LGL-1 as a probe is therefore similar to that found using the 639-bp probe of Ly-49 (15). The overall results of our Southern blotting procedures indicate that while there are a number of Ly-49–related genes in the mouse, there may not be any genes with a high degree of homology in the rat or human genome. If there are any homologous genes in humans, they apparently are much more evolutionarily divergent.
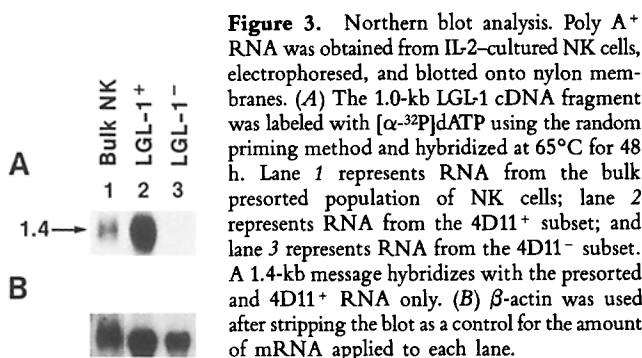
LGL-1 versus Ly-49 Subset Analysis of NK Cells. FCA of highly enriched populations of B6 splenic NK cells reveals four distinct subsets of cells when stained for LGL-1 and Ly-49. Fig. 5 *A* demonstrates that 32% of the cells are LGL-1$^+$Ly-49$^-$ (*quadrant 1*), 8% LGL-1$^+$Ly-49$^+$ (*quadrant 2*), 53% LGL-1$^-$Ly-49$^-$ (*quadrant 3*), and 7% LGL-1$^-$Ly-49$^+$ (*quadrant 4*). Because the number of NK-1.1$^+$ cells after enrichment was ∼75%, the LGL-1$^-$Ly-49$^-$ population also contains these non-NK cells. Previous data from this labora-

Figure 3. Northern blot analysis. Poly A$^+$ RNA was obtained from IL-2–cultured NK cells, electrophoresed, and blotted onto nylon membranes. (*A*) The 1.0-kb LGL-1 cDNA fragment was labeled with [α-^{32}P]dATP using the random priming method and hybridized at 65°C for 48 h. Lane *1* represents RNA from the bulk presorted population of NK cells; lane *2* represents RNA from the 4D11$^+$ subset; and lane *3* represents RNA from the 4D11$^-$ subset. A 1.4-kb message hybridizes with the presorted and 4D11$^+$ RNA only. (*B*) β-actin was used after stripping the blot as a control for the amount of mRNA applied to each lane.
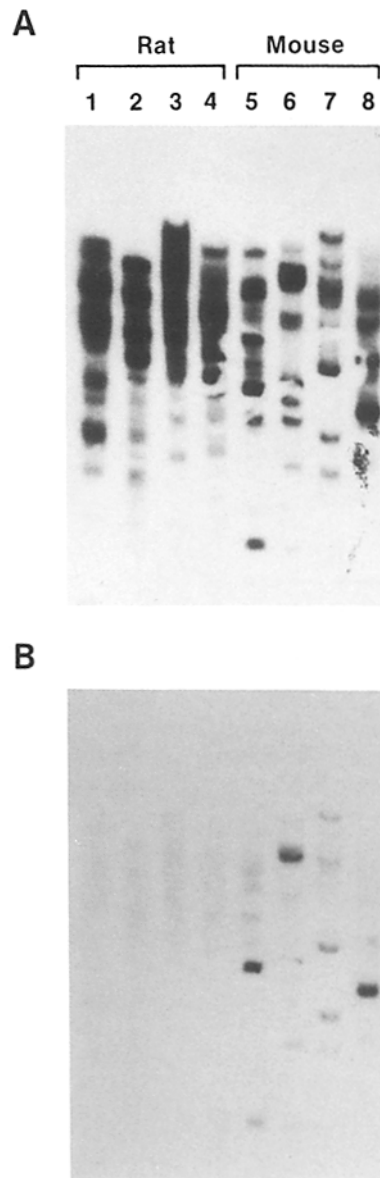

Figure 4. Southern blot analysis. DNA isolated from rat and mouse spleen was digested with the following restriction enzymes: lanes *1* and *5*, HindIII; lanes *2* and *6*, PvuI; lanes *3* and *7*, SstI; and lanes *4* and *8*, EcoRI. Digests were applied to gels, electrophoresed, and blotted onto nylon membranes. Hybridization with the 1.0-kb LGL-1 fragment after labeling with [α-^{32}P] dATP was performed at 42°C. Blots were washed under either nonstringent (50°C; *A*) or stringent (65°C; *B*) conditions to determine the relative degree of homology between the two species.
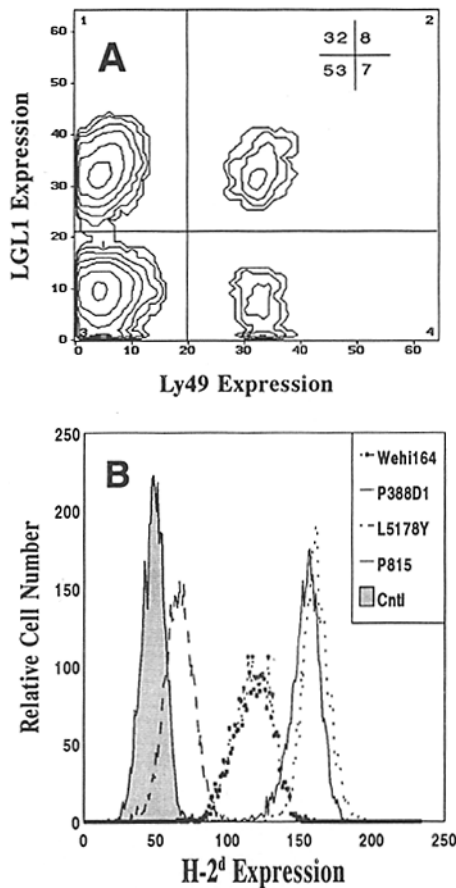
Figure 5. (A) FCA of LGL-1 versus Ly-49 on splenic B6 NK cells. Freshly isolated splenic NK cells were enriched from B6 mice after nylon wool nonadherent cells were depleted of CD4$^+$ and CD8$^+$ cells by antibody + c' followed by panning to remove residual B cells. The resulting cells were >75% NK-1.1$^+$. NK cells were stained with mAb A1, washed, and sandwiched with a FITC-αmouseγ2A + mAb 4D11 PE. FITC-labeled cells (Ly-49$^+$) and PE-labeled cells (LGL-1) were analyzed on a FACScan® and divided into quadrants based on staining with control antibodies. Quadrant 1, LGL-1$^+$ Ly-49$^-$ = 32%; quadrant 2, LGL-1$^+$Ly-49$^+$ = 8%; quadrant 3, LGL-1$^-$ Ly-49$^-$ = 53%; and quadrant 4, LGL-1$^-$Ly-49$^+$ = 7%. (B) H-2^d expression of tumor target cells. Tumor targets were prepared for FCA by staining cells with anti-H-2D^d (clone 34-2-12) followed by FITC goat anti–mouse γ 2A. A and B are representative of at least three similar determinations.

tory has shown that LGL-1$^+$ cells do not lyse selected tumor target cells such as P815 and L5178Y, both of which express high levels of class I (H-2D^d) (18). However, examination of a panel of tumor targets for a correlation between H-2^d expression and resistance to lysis by LGL-1$^+$ cells was not very strong. Since Ly-49$^+$ cells do not lyse H-2^d or H-2^k targets, the Ly-49$^+$ subset of NK cells (~20%) within the LGL-1$^+$ (~40%) population could represent a major effector cell subset. Therefore, Ly-49$^+$LGL-1$^+$ cells might explain to a certain degree the poor lysis of H-2^d–bearing targets by LGL-1$^+$ cells. Our observation that ~25% of the total LGL-1$^+$ cells coexpress Ly-49 indicated that further subset analysis would be necessary to evaluate the lytic potential of these NK cell subsets.

Functional Analysis of LGL-1$^+$ versus Ly-49$^+$ NK Cells. To further examine the functional properties of the LGL-1 and Ly-49$^+$ subsets, it was necessary to separate the subsets expressing either LGL-1 or Ly-49 alone from those expressing both phenotypic markers (LGL-1$^+$Ly-49$^+$). Splenic NK cells from B6 mice were enriched, stained, sorted into the following subsets, and cultured in high-dose IL-2: total Ly-49$^+$ cells (18%), LGL-1$^+$Ly-49$^-$ (35%), and LGL-1$^-$Ly-49$^-$ (47%). Table 1 confirms previously published data that the total Ly-49$^+$ subset of NK cells does not lyse the majority of targets expressing H-2D^d. However, both P338D1 and Raw 264, which express low to zero levels of H-2D^d, are lysed somewhat more efficiently than the other H-2^d targets. In addition, the LGL-1$^+$ subset of NK cells, upon removal of any Ly-49$^+$ cells (LGL-1$^+$Ly-49$^-$), did not lyse target cells that express very high levels of H-2^d, such as P815 and L5178Y. Tumor targets expressing somewhat lower levels of H-2^d, such as WEHI-164 and more notably P388D1 and Raw 264 (Fig. 5 B), are lysed much more efficiently by the LGL-1$^+$ cells. NK cells lacking expression of LGL-1 and Ly-49 appear to be quite capable of lysing all tumor cells examined. Of interest, this LGL-1$^-$Ly-49$^-$ subset actually contains enhanced levels of cytotoxicity against these tumor targets compared with the presorted population. Therefore, NK cells expressing the LGL-1 phenotype are similar, but not identical, to Ly-49$^+$ cells in their inability to lyse H-2^d tumor targets. The inhibition by tumor targets, however, appears to be generally associated with high levels of H-2^d expression.

The lysis of tumor cells, although modulated by the expression of class I MHC molecules, is likely to be influenced by other adhesion/triggering molecules as well. We therefore used normal Con A lymphoblasts (24) to further examine the role of LGL-1$^+$ and Ly-49$^+$ cells in the lysis of class I–expressing target cells. The data presented in Fig. 6 show that NK cells expressing either LGL-1 or Ly-49 are not capable of lysing tumor targets expressing high levels of H-2^d (Fig. 6 A) or normal Con A lymphoblasts from BALB/c mice that are H-2^{d+} (Fig. 6 D). NK cells expressing LGL-1 and/or Ly-49 are fully capable of lysing tumor target cells expressing H-2^b (Fig. 6 B) and Con A lymphoblasts from C57BL/6 mice that are H-2^{b+} (Fig. 6 C). These results support the hypothesis that both the LGL-1 and Ly-49 molecules transmit negative signals for lysis of H-2^d–expressing targets.

mAbs 4D11 and A1 Overcome Inhibitory Effects of Class I. To ascertain whether the LGL-1 and Ly-49 molecules on the surface of NK cells are directly involved in class I inhibition of lysis, antibody-blocking assays have been used. Using NK subsets expressing either the LGL-1$^+$ or Ly-49$^+$ markers alone, or in combination, cytotoxicity assays were performed in the presence of various antibodies. We used the tumor targets P815 (H-2^{d+} and FcγRII$^+$) and WEHI-164 (H-2^{d+} but FcγRII$^-$). Both types of targets were used to ensure that altered levels of lysis was not caused by a reverse-type ADCC (RADCC) mechanism.

Table 2, A and B, presents the results of the lytic data obtained when mAb to LGL-1 (4D11), Ly-49 (A1), and the NK-

Table 1. *Lytic Function of LGL-1⁺ versus Ly-49⁺ NK Cells*

NK subsets	YAC (a)	P815 (d-4⁺)	L5178Y (d-4⁺)	WEHI-164 (d-3⁺)	P388D1 (d-1⁺)	Raw 264 (d-o)	EL-4 (b)	MBL-2 (b)
Presorted	2,600	55	41	413	2,860	1,175	394	569
Ly-49⁺ (total)	1,524	1	<1	9	1,641	139	409	315
LGL-1⁺Ly-49⁻	2,656	4	6	293	2,412	1,371	424	442
LGL-1⁻Ly-49⁻	5,013	247	250	795	4,816	4,459	476	722

NK cells were enriched from C57BL/6 spleens and stained for LGL-1 and Ly-49. Cells were sorted into subsets containing total Ly-49⁺ cells (18%), LGL-1⁺Ly-49⁻ cells (35%), and LGL-1⁻Ly-49⁻ cells (47%) and cultured in high-dose IL-2 for 7 d. At day 7, the subsets were stained and examined for LGL-1 and Ly-49 expression as follows: presorted (LGL-1 = 60%, Ly-49 = 23%); total Ly-49⁺ (LGL-1 = 65%, Ly-49 = 97%); LGL-1⁺Ly-49⁻ (LGL-1 = 98%, Ly-49 = 0); and LGL-1⁻Ly-49⁻ (LGL-1 = 2%, Ly-49 = 1%). Cells were washed twice before assaying. Tumor target cells were labeled with ⁵¹Cr and cytotoxicity assays performed at E/T ratios starting at 20:1. LU at 30% per 10⁷ cells are presented. Letters and numbers in parentheses represent the H-2 haplotype of the target and its relative degree of expression by FCA on a 0–4⁺ scale. This is a representative experiment of at least three similar assays performed.

1.1 (PK136) markers were added to 4-h cytotoxicity assays against either P815 or WEHI-164. These results demonstrate that addition of mAb 4D11, A1, or 4D11 + A1 enhances lysis against both FcγRII⁺ P815 and FcγRII⁻ WEHI-164 targets when the effector cells express either LGL-1, Ly-49, or both markers simultaneously. The specificity of this response was shown by the increased lysis observed with LGL-1⁺Ly-49⁻ cells upon addition of mAb 4D11 (anti–LGL-1), but not by mAb A1 (anti–Ly-49). A more pronounced effect is seen with cells expressing both LGL-1 and Ly-49, where

addition of mAb 4D11 + A1 produces a synergistic response in lysing P815. Similar specificity is provided in the LGL-1⁻Ly-49⁻ subset, which does not respond with enhanced lysis to addition of either mAb 4D11 or A1. These results indicate that class I inhibition seen with LGL-1⁺ cells can be reversed by addition of mAb 4D11, and they confirm the data of Karlhofer et al. (7) that mAb A1 can reverse class I inhibition mediated by Ly-49⁺ NK cells.

Antibodies to H2-Dᵈα₁α₂ and H2-Lᵈα₁α₂ Reverse Inhibition of LGL-1⁺ NK Cells. To confirm that inhibition of cyto-

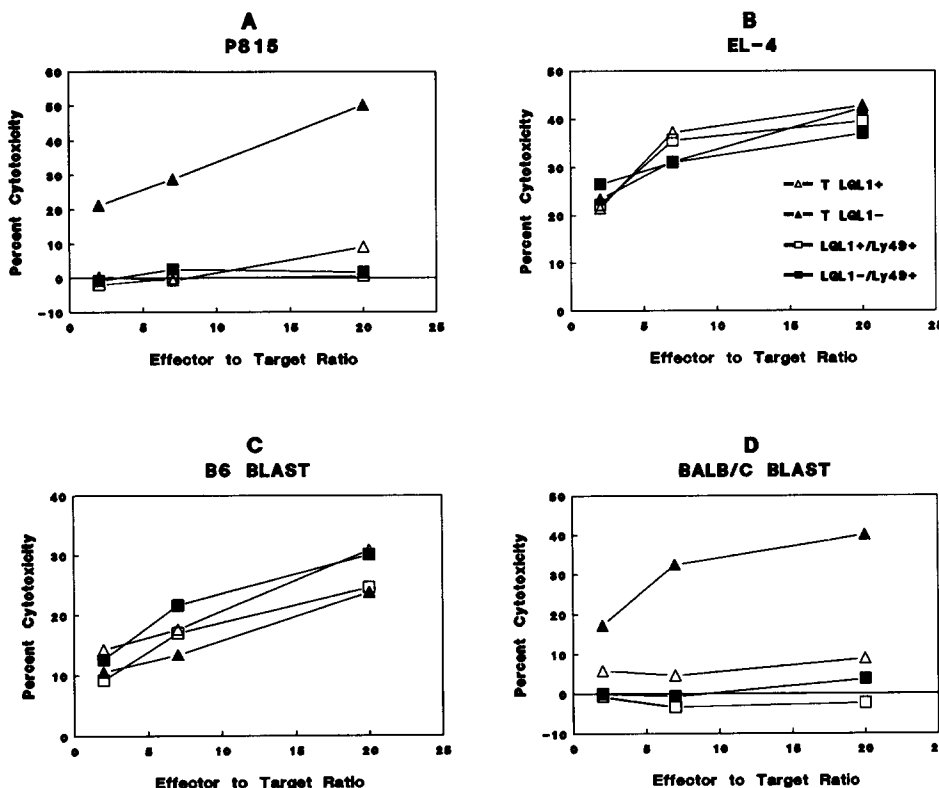**A P815**

B EL-4

C B6 BLAST

D BALB/C BLAST

Figure 6. Lysis of Con A lymphoblasts and tumor targets by LGL-1⁺ and Ly-49⁺ NK cell subsets. NK cells were enriched from B6 spleens, stained with mAb A1 followed by FITC α Mγ2A plus 4D11 PE, and sorted into the respective subsets: total LGL-1⁺ (43%); total LGL-1⁻ (57%); LGL-1⁺Ly-49⁺ (11%); and LGL-1⁻Ly-49⁺ (8%). After culture in 10³ U/ml IL-2 for 6 d, the cells were washed twice and assayed. All subsets were ≥95% pure for their respective markers at the time of assay. Target cells were labeled for 1.5 h with ⁵¹Cr. NK cells were assayed against target cells at E/T ratios of 20:1, 7:1, and 2:1, and results were expressed as percentage of cytotoxicity. This is a representative experiment of at least three similar assays performed.

Table 2. *mAb to LGL-1 and Ly-49 Overcome Class I Inhibition of NK Cells*

mAb	LGL-1⁺Ly-49⁻	LGL-1⁺Ly-49⁺	LGL-1⁻Ly-49⁻	LGL-1⁻Ly-49⁺
	NK Cell Subsets vs P815			
Media	409	15	1,429	34
Rγ2A (control)	376	16	1,589	36
4D11	2,144	39	1,310	44
Ly-2.2 (control)	239	13	892	32
PK136	1,200	38	2,016	66
A1	354	52	800	2,548
4D11 + A1	2,144	1,931	1,193	3,123
	NK Cell Subsets vs WEHI-164			
Media	1,675	82	2,513	61
Rγ2A	1,708	67	2,629	70
4D11	3,501	127	2,611	64
Ly-2.2	1,032	55	2,456	62
PK136	1,355	67	2,084	45
A1	1,256	400	2,213	1,253
4D11 + A1	3,788	2,283	2,263	1,139

NK cells were enriched from B6 spleens, stained with 4D11, PE, and A1⁺ FαMγ2A, and sorted into the respective subsets: LGL-1⁺Ly-49⁻ (32%); LGL-1⁺Ly-49⁺ (11%); LGL-1⁻Ly-49⁻ (47%); and LGL-1⁻Ly-49⁺ (9%). After culture in 10^3 U/ml IL-2 for 6 d, the cells were washed twice and assayed. P815 and WEHI-164 were labeled with ⁵¹Cr. NK cells were assayed against P815 at E/T ratios starting at 10:1 and against WEHI-164 at E/T ratios starting at 3:1. mAbs were added to the cytotoxicity assay at a final concentration of 2 μg/well and remained for the 4-h assay. Results are expressed at LU 30% per 10^7 cells. Rγ2A is a negative isotype control mAb for 4D11, and Ly-2.2 is a negative isotype control mAb for A1 and PK136. These results are representative of at least three similar assays.

toxicity by P815 targets was caused by the presence of class I on their surface, we performed cytotoxicity assays in the presence of various class I antibodies. Data presented in Fig. 7 indicate that two class I proteins may be recognized by LGL-1 and inhibit cytotoxicity. Antibodies to H2-Lᵈ and H2-Dᵈ were very effective at enhancing cytotoxicity against P815
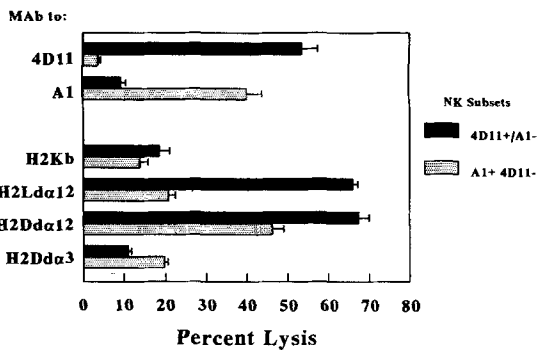

Figure 7. Antibodies to H2-Lᵈ and H2-Dᵈ enhance cytotoxicity of P815 targets by LGL-1⁺ cells. NK cells from B6 mice were enriched, sorted into LGL-1⁺Ly-49⁻ and Ly-49⁺LGL-1⁻ subsets, and cultured for 6–10 d in IL-2. After washing twice, NK cells were tested against P815 targets at an E/T ratio of 10:1 in a 4-h ⁵¹Cr release assay. Various antibodies to either LGL-1, Ly-49, or a variety of class I proteins were included at a final concentration of 1–2 μg per well. Each group consisted of four replicates; the SEM are denoted by the error bars. This is one of two representative experiments performed.

target cells. LGL-1⁺Ly-49⁻ NK cells showed baseline cytotoxic activity between 10 and 20%, depending on the particular control antibody added. When antibodies to either H2-Lᵈ or H2-Dᵈ were added, however, cytotoxicity increased to 65–70%. It must be noted that only antibodies to the $\alpha_1\alpha_2$ domains of H2-Lᵈ and H2-Dᵈ were effective at enhancing the cytotoxic response of these cells. Antibodies to the H2-Dᵈ (α_3 domain) and the H2-Lᵈ (α_3 domain) were not capable of enhancing cytotoxicity (data not shown). Ly-49⁺LGL-1⁻ cells demonstrated enhanced cytotoxicity of P815 targets only in response to the addition of αH2-Dᵈ$\alpha_1\alpha_2$, as demonstrated in previous studies (7). The data in Fig. 7 strongly implicate class I expression of H2-Lᵈ and H2-Dᵈ as inhibitory molecules that may bind to the LGL-1 receptor on NK cells. Similar experiments with Con A lymphoblasts from B6 versus BALB/c mice demonstrated almost identical results to those seen with P815. LGL-1⁺Ly-49⁻ NK cells were not able to kill BALB/c blasts alone, but upon addition of antibodies to H2-Dᵈ$\alpha_1\alpha_2$ and H2-Lᵈ$\alpha_1\alpha_2$, cytotoxicity was greatly augmented when compared with control antibodies (data not shown). Data obtained using antibodies to both the effector cells (4D11) and target cells (H2-Dᵈ$\alpha_1\alpha_2$ and H2-Lᵈ$\alpha_1\alpha_2$) provide strong evidence for the involvement of H2-Dᵈ and H2-Lᵈ as ligands for the LGL-1 receptor molecule. Significant differences are therefore observed in the binding of class I molecules by LGL-1 and Ly-49. Ly-49 recognizes H2-Dᵈ$\alpha_1\alpha_2$ and H2-Dᵏ, whereas LGL-1 recognizes both H2-Lᵈ$\alpha_1\alpha_2$ and H2-Dᵈ$\alpha_1\alpha_2$.

Discussion

The cloning and sequencing of the murine LGL-1 cDNA has revealed it to be a member of the Ly-49 gene family, which belongs to the C-type lectin family of type II transmembrane proteins. This group of proteins includes NKR-P1, the asialoglycoprotein receptor, the low affinity receptors for IgE (FcεRII) (25), and the CD69 early lymphocyte activation gene (26). Another NK-specific protein on murine NK cells, SW5E6, has recently been found to be an Ly-49 family member. The high degree of homology observed between the LGL-1 and Ly-49 proteins is most notable in that they share 10 of 11 extracellular cysteines. This high percentage of cysteines accounts for the highly disulfide-linked nature of these proteins when analyzed on SDS-PAGE and further suggests similar functional characteristics. It also must be mentioned that recent crosshybridization assays by Smith et al. (27) using Ly-49 cDNA has resulted in the detection of a number of Ly-49–related genes, including one that appears to be nearly identical to LGL-1. Therefore, using the nomenclature of Yokoyama and coworkers, LGL-1 is Ly-49G2, 5E6 is Ly-49C, and Ly-49 is Ly-49A. It must be noted, however, that LGL-1 is only 97% homologous to the Ly-49G2 cDNA isolated from B6 mice, and may therefore represent the C.B.17 SCID form of Ly-49G2.

Ly-49G2 and Ly-49A are restricted in their lymphoid distribution, and are predominantly found to reside on subsets of murine NK cells. Since Ly-49 has been shown to function as an inhibitor of lysis when in contact with class I molecules (H-2D^d, H-2D^k), we were interested in examining LGL-1$^+$ cells for similar functional characteristics. Previous data from this laboratory indicated that the LGL-1 molecule was a potential triggering molecule on NK cells, as it appeared to mediate RADCC (21). Since mAb 4D11 was capable of enhancing lysis of P815 target cells (FcγR11$^+$), we believed this to be mediated by a RADCC-type mechanism. However, mAb 2.4G2 against the FcγRII was not able to block 4D11-induced lysis, but could block RADCC induced by mAb PK-136. These results suggested that alternative mechanisms may exist for the enhanced lysis of P815 target cells when using mAb 4D11. The results of our present studies indicate that the LGL-1 molecule on NK cells, when bound to its appropriate ligand, may initiate an inhibitory signal in NK cells, as seen with Ly-49. When we analyze either total LGL-1$^+$ cells or LGL-1$^+$Ly-49$^-$ NK cells, there appears to be a selective inhibition of lysis that correlates with the degree of expression of H-2D^d on target cells. This inhibition of lysis is not limited to tumor cell targets. Normal Con A lymphoblasts from BALB/c (H-2^d), but not B6 (H-2^b), mice are relatively resistant to lysis by LGL-1$^+$ NK cells. We also have performed lytic assays with LGL-1$^+$ and LGL-1$^-$ cells against BALB/c × C57BL/6 F1 Con A lymphoblasts, in which lysis is greatly reduced in LGL-1$^+$ subset (data not shown). Addition of mAb 4D11 to assays in which LGL-1$^+$ cells are tested against either P815 (FcγRII$^+$) or WEHI-164 (FcγRII$^-$) targets results in enhanced lysis of both targets when compared with control antibodies. This data suggests that the LGL-1 molecule, when blocked by the 4D11 antibody, is no longer able to deliver negative signals from H-2^{d+} target cells. The results of these studies suggest that the LGL-1 molecule may interact with class I H-2^d or a closely related molecule on the target cell membrane.

In an attempt to further characterize the LGL-1 ligand, cytotoxicity assays were performed in which LGL-1$^+$ cells were assayed against P815 and Con A blasts in the presence of various antibodies to class I molecules. Our data suggest that LGL-1 recognizes both H-2D^d and H2-L^d as inhibitory molecules on target cells. Of interest is the fact that only antibodies to the $\alpha_1\alpha_2$ peptide-binding region of these class I molecules are able to overcome their inhibitory effects. Antibodies recognizing the α_3 domains of H2-D^d or H2-L^d have little or no effect on cytotoxicity. These findings are important in two respects. First, they establish a unique specificity for LGL-1$^+$ NK cells in recognizing H2-D^d and H2-L^d. This specificity appears to be distinct from that of Ly-49A, which recognizes H2-D^d and H2-K^d. Second is the relative importance of the $\alpha_1\alpha_2$ domain of these class I molecules. Since both Ly-49 and LGL-1 appear to be inhibited by the peptide-binding region of class I molecules, it emphasizes the potential significance of the peptides presented in the binding domain of $\alpha_1\alpha_2$. This is particularly relevant to LGL-1 because of its limited inhibition by H-2D^d–expressing tumor targets. The ability of class I molecules on target cells to inhibit cytotoxicity by LGL-1$^+$ cells may be governed by the degree of class I expression, unique peptides presented by a particular cell type, or differential glycosylation of these class I molecules.

Transfections of different class I molecules into class I$^-$ target cells also will help to further define the appropriate ligand for LGL-1. Recent work by Kane (28) has demonstrated that Ly-49 is capable of binding to H2-D^d and H2-D^k in a density-dependent manner. These results, along with the fact that there appear to be a number of Ly-49 family members (15, 29), suggest that these proteins may represent a group of molecules with different affinities for class I. Studies by Yokoyama et al. (15) have demonstrated that there are at least five RFLP patterns in inbred mice that are displayed by Ly-49 family members, and all appear to reside on chromosome 6. One question raised by these data is why more than one molecule would be present on different NK cell subsets that might recognize the same class I molecule, albeit with different affinities. It will also be interesting to wait for the development of mAbs to the other members of the Ly-49 family to see if they have similar functional attributes. Further studies also are necessary to determine why Ly-49 and possibly LGL-1 are inhibited by class I molecules of allogeneic rather than syngeneic animals. Even if the fine specificity of binding is found to reside in the peptides that occupy the class I molecules (4, 8), it would be expected that if this protein–ligand interaction was to avoid lysis of "self," it would be present in a syngeneic environment. Only further characterization of the members of the Ly-49 gene family and the development of mAbs to their respective proteins will help to define their true physiological roles.

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