Molecular Cloning of gp42, A Cell-Surface Molecule That Is Selectively Induced on Rat Natural Killer Cells by Interleukin 2: Glycolipid Membrane Anchoring and Capacity for Transmembrane Signaling

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Summary

We have previously shown that in vitro culture of rat natural killer (NK) cells in high concentrations of recombinant interleukin 2 (rIL-2) leads to the expression of a surface glycoprotein with a molecular mass of ~42 kD. This glycoprotein, gp42, is not induced on other lymphocytes and thus provides a lineage-specific marker for rIL-2–activated NK cells. We here present the nucleotide sequence for gp42 cDNA. The open reading frame encodes 233 amino acids with three potential sites for N-linked glycosylation. The deduced amino acid sequence lacks an apparent transmembrane domain and instead contains a hydrophobic COOH terminus that is characteristic of glycosyl-phosphatidylinositol (GPI)-anchored surface proteins. Consistent with this, gp42 is cleaved from the NK-like cell line, RNK-16, by phosphatidylinositol-specific phospholipase C (PI-PLC), as is gp42 expressed on CHO cells that have been transformed with gp42 cDNA. On rIL-2–activated NK cells, gp42 is resistant to PI-PLC, though our studies suggest that gp42 on these cells is still expressed as a GPI-anchored molecule.

Antibody to gp42 stimulates in RNK-16 cells an increase in inositol phosphates and in intracellular calcium, signals that are associated with the activation of lymphocytes, including NK cells. rIL-2–activated NK cells, however, lack this response to gp42 as well as to other stimuli. Thus, gp42, the only NK-specific activation antigen, is a GPI-anchored surface molecule with the capacity to stimulate transmembrane signaling.

Natural killer (NK) cells are large granular lymphocytes that spontaneously lyse certain tumor cells and cell lines (1–3). NK cells express relatively high levels of the p75 component of the IL-2 receptor (4), and they readily respond to IL-2, as assessed by proliferation (5), by an increase in cytotoxicity (5, 6), by increased production of serine proteases (7), and by a rapid increase in the transcription and translation of genes for at least several important cytokines, including IFN-γ, TNF, and granulocyte/macrophage (GM)[1]-CSF (5, 8–10).

The IL-2–induced differentiation of NK cells is associated with the expression of several cell-surface molecules that are also expressed by activated T lymphocytes, such as the p55 subunit of the IL-2 receptor (CD25, reference 11) and CD69 (Leu-23, reference 12). Recently, we described a rat cell surface glycoprotein that is not detectable on freshly prepared lymphocytes, including NK cells, but that is selectively expressed on NK cells when they are cultured in high concentrations of rIL-2 (13). The molecule, gp42, is expressed within 6–8 d on all rIL-2-activated NK cells. It is also present on the rat NK-like leukemia cell line, RNK-16, where it was originally identified (13). It is not, however, induced on T cells by either IL-2 or mitogens. Thus, gp42 appears to be an activation molecule that is selectively expressed by the NK cell lineage.

We have isolated cDNAs for gp42 from rIL-2–activated rat NK cells and here present the nucleotide sequence and the deduced amino acid sequence. Neither sequence shows significant homology with known proteins. Interestingly, the sequence contains no putative transmembrane domain and instead has features characteristic of proteins attached to the cell surface membrane by a glycosyl-phosphatidylinositol (GPI) anchor (14, 15). Several other GPI-anchored proteins on lymphocytes can stimulate the intracellular release of inositol phosphates (InsPs) and a rise in intracellular free calcium ($[Ca^{2+}]_i$), signals that are associated with lymphocyte activation. We find that these signals are also generated within RNK-16 cells by mAbs to gp42.

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; GM, granulocyte/macrophage; GPI, glycosyl-phosphatidylinositol; InsPs, inositol phosphates; PI-PLC, phosphatidylinositol-specific phospholipase C.

Materials and Methods

Antibodies. The mAbs against gp42 have been described (13). Except where noted, the anti-gp42 mAb used in these studies was from hybridoma 3G7 (mouse IgG2a). mAbs OX-18 (anti-rat class I MHC antigen; reference 16), OX-34 anti-rat CD2; reference 17), and OX-45 (a GPI-anchored surface protein on rat leukocytes; reference 18) were kindly provided by Dr. Alan Williams, Oxford University, Oxford, UK. For fluorescent staining, mAbs were either directly conjugated with fluorescein by published methods (19, 20) or were identified by a second-step antibody (fluorescein-conjugated goat anti-mouse Ig; Cappel Laboratories, Malvern, PA). Studies involving the cross-linking of mouse mAbs used affinity-purified goat anti-mouse Ig (Cappel Laboratories).

Cells and Cell Culture. RNK-16 cells were adapted for in vitro growth as previously described (20) and were carried in RPMI 1640 (without IL-2) + 10% FCS, L-glutamine (0.3 mg/ml), penicillin-K (100 U/ml), and streptomycin sulfate (0.1 mg/ml). rIL-2–activated NK cells were generated by a modification of the method of Vujanovic et al. (21). Briefly, cells were incubated in RPMI 1640, supplemented with 10% FCS, 2 mM glutamine, 5×10^{-5} M 2-ME, streptomycin, penicillin, and 1,000 U/ml rIL-2 (Cetus Corp., Emeryville, CA). After 72 h, the nonadherent cells were discarded, fresh medium was added (including rIL-2), and incubation was continued for another 72 h. The cells were harvested by the addition of PBS containing 0.5 mM EDTA. Chinese hamster ovary (CHO) cells were carried in Ham's F12 medium, supplemented as for RPMI 1640. After transformation, the cells were carried in the same medium without hypoxanthine (using dialyzed FCS).

Molecular Cloning of gp42 cDNA. Total cellular RNA was prepared from 5×10^8 rat rIL-2–activated NK cells by the guanidine isothiocyanate method (22). From this, poly(A)$^+$ RNA was isolated by absorption on oligo-dT-cellulose beads and used to generate a cDNA library by the method of Gubler and Hoffman (23). After methylation of internal EcoRI sites, EcoRI linkers were attached and the library was ligated into λgt11 arms (Amersham Corp., Arlington Heights, IL). Initial cloning efficiency was 3×10^6 PFU/μg cDNA. 3×10^5 PFU were plated on Y1090 cells, and expression was induced with IPTG-impregnated nitrocellulose filters. The denatured filters were screened with a combination of two anti-gp42 mAbs, 3G7 and 6D8 (13). In brief, the filters were washed in distilled water and then incubated for 2 h at room temperature in PBS containing powdered milk (1 g/100 ml). The filters were incubated overnight at 4°C with the gp42 mAbs at a concentration of 10 μg/ml each in PBS/powdered milk. The filters were then washed twice in PBS containing TWEEN-20 (0.05 g/100 ml). For detection of bound mAbs, the filters were incubated for 2 h at 4°C with affinity-purified goat antibody to mouse Ig that had been conjugated to alkaline phosphatase (Calbiochem-Behring Corp., San Diego, CA), at a final concentration 10 μg/ml in PBS. The filters were washed twice in PBS/TWEEN as before and then exposed to nitroblue tetrazolium chloride (0.34 mg/ml) and 5-bromo-4-chloro-3-indolylphosphate-*p*-toluidine salt (0.165 mg/ml) for 30 min in the dark at room temperature in 0.1 M Tris·HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$ (Protoblot® buffer, Promega Biotec, Madison, WI). The filters were rinsed in water before examination for stained colonies.

Of 36 clones identified, 11 were selected for subcloning, and 7 of these remained positive through three rounds of subcloning. The phage were partially purified by immunoabsorption using Lambdasorb® (Promega Biotec), according to the method of the manufacturer, before the preparation of phage DNA (24). The purified phage DNA was then used as a substrate for amplification of the cDNA by the PCR (25). The primers were oligonucleotides corresponding to flanking sequences in the λgt11 *lacZ* gene. The oligonucleotides were prepared with flanking ClaI sites, providing an option for releasing the intact insert if the cDNAs contained internal EcoRI. The amplified product thus included both the cDNA and adjacent sequences from the *lacZ* gene, assuring that the amplified sequences represented the inserted cDNA. For amplification, 10 ng of substrate phage DNA was exposed to 25 pM of each primer and 1.0 U *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer/Cetus Corp., Emeryville, CA) for 25 cycles in a volume of 50 μl buffer (67 mM Tris·HCl, 6.7 mM $MgCl_2$, 16.6 mM $[NH_4]_2SO_4$, 1.6 mM dNTPs, 170 μg/ml BSA), mixed with 5 μl DMSO. The cycles included 1.5 min denaturation at 95°C, 1 min annealing at 45°C, and 2 min enzymatic primer extension at 72°C.

Northern Blotting. Northern blotting utilized amounts of total cellular RNA or of poly(A)$^+$ RNA as indicated. To further assure equal loading with RNA, the replicate samples were loaded on the same gel, and one was examined by staining with ethidium bromide. The other was transferred to nitrocellulose membranes (0.45 μm; BioRad Laboratories, Richmond, CA). The RNA-containing gels were washed twice in 10× SSC for 20 min per wash. Nitrocellulose filters were soaked in 20× SSC, and the RNA was transferred overnight using a 20× SSC bath. Filters were baked at 80°C for 2 h and then incubated (65°C for 20–30 min, then 45°C for 3 h) in hybridization buffer (50% vol/vol formamide, 5× SSC, 0.5% wt/vol SDS, 1× Denhardt's solution, and 100 μg/ml salmon sperm DNA). The ^{32}P-labeled probe ($1–3 \times 10^7$ cpm) was added in a volume of 10 μl and hybridized overnight at 42°C. The filters were washed twice for 15 min at 42°C in 1× SSC + 0.1% SDS, then twice for 15 min at 42°C in 0.25× SSC + 0.1% SDS, before exposure to radiographic film.

Sequencing. For further analysis, including sequencing, the clones were ligated in both orientations into the phagemid, Bluescript SK^{-R} (Stratagene, San Diego, CA). For sequencing, ssDNA from two independently derived clones was prepared from transformed bacteria by rescue with R408 helper phage, according to the method of the supplier (Stratagene). Sequencing of both strands was initiated by using the Sequenase® variant of T7 DNA polymerase (U.S. Biochemical Corp., Cleveland, OH) for dideoxy (chain termination) sequencing (26). New oligomers, a total of 15, were synthesized as needed to prime overlapping sequences.

The nucleotide sequence of gp42 was compared with sequences in GenBank (release 61, rechecked on release 64). The deduced amino acid sequence was compared with sequences in Swiss-Prot (release 11) and with deduced amino acid sequences obtained by translation of part of the GenBank database. Searches were performed by using the FASTDB program (Intelligenetics, Inc., Mountain View, CA) on a Sun Workstation. Amino acid domain searches were performed with a unitary matrix, a k-tuple of 2, and a word length of 32. Additional alignments were analyzed with the PEP program (Intelligenetics, Inc.).

Transformation of CHO Cells. For transformation of CHO (*dhfr*$^-$) cells, cDNA from one clone (clone 2 in Fig. 2) was subcloned into the vector pXM (27). The cells were transformed by electroporation, using 10^7 cells in 1 ml phosphate-buffered sucrose (9 gm/100 ml) containing 10 μg of vector, using a field strength of either 300 V or 330 V/0.4 cm with a 25 μFarad capacitor. The vector pXM contains the gene for dihydrofolate reductase (*dhfr*), permitting selection in the absence of hypoxanthine.

Treatment of Cells with PI-specific Phospholipase C (PI-PLC). To cleave surface proteins attached by a GPI-anchor (14, 15), 10^7 cells were incubated for 1 h at 37°C in 1 ml PBS + BSA (1 μg/100 ml) containing 0.5 U of PI-PLC from *Bacillus thuringiensis* (ICN

KBK Laboratories Inc., Plainview, NY). Cells were subsequently stained for the expression of gp42 or OX-45. By these methods, the concentration of PI-PLC used provided maximum release of gp42 from RNK-16 cells.

Measurement of InsPs. InsPs were resolved and quantified as we have described (20), by labeling cells with [^{3}H]inositol (20–40 μCi/ml) for 3 h, before stimulation by mAbs. At intervals after stimulation, aliquots of cells (2–5 × 10^6) were rapidly pelleted then lysed in cold 10% TCA. After removal of insoluble material, the supernatant was extensively extracted with diethyl ether. Labeled InsPs were resolved by sequential elution using anion-exchange chromatography with Dowex-1X8 (100–200 mesh) in the formate form, as described (20). Alternatively (as indicated) total InsPs were co-eluted in 6 ml of 2 M ammonium formate, 0.1 M formic acid.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was quantified by monitoring fluorescence of cells loaded with the calcium-sensitive fluor, indo-1, as we have described (20).

Surface Labeling, Immunoprecipitation, and SDS-PAGE. These were performed as we have described (13).

Results

Identification and Characterization of the cDNA for gp42. Seven cDNA clones were isolated by screening a λgt11 library prepared from rIL-2–activated NK cell mRNA. Of these, five clones were similar in size (~1.2 kb) and gave identical patterns on gel electrophoresis after digestion with MboI. Initial evidence that these clones represented gp42 came from Northern blot analysis using one of the clones: no message could be detected in freshly prepared spleen cells, but a single message, ~1.4 kb, was expressed at high levels in rIL-2–activated NK cells (Fig. 1). Message was also absent from whole cell RNA prepared from heart, kidney, or liver (not shown).

The cDNA from two independently derived clones was sequenced. Both strands from one clone were sequenced in their entirety. For the other, one strand was sequenced in its entirety, and the second strand was sequenced in portions

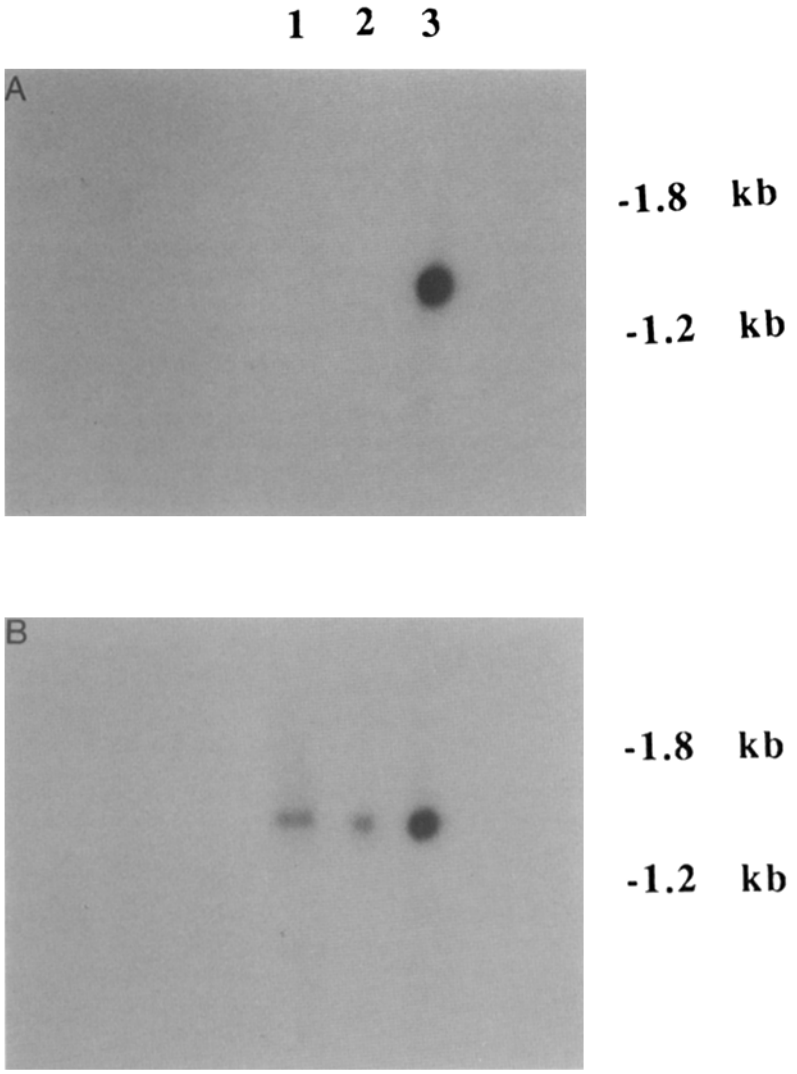

Figure 1. The cDNA for gp42 identifies message that is expressed in rIL-2–activated NK cells but not in freshly prepared spleen cells. Northern blots were probed with ^{32}P-labeled cDNA from clone 2 (*A*) or, as a control, with cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, reference 60) (*B*). (Lane *1*) poly(A)$^+$ RNA from fresh spleen cells, 1 μg. (Lane *2*) Whole cell RNA from fresh spleen cells, 10 μg. (Lane *3*) Whole cell RNA from rIL-2–activated NK cells, 10 μg. The blot was first probed with cDNA for gp42 and then stripped before reprobing with GAPDH. Successful removal of the first probe was documented by exposure to film for 48 h, while the probed blots were exposed only overnight. Message for gp42 is not detectable in fresh spleen cells but is >100-fold above background in rIL-2–activated NK cells (*A*, lane *3*). Message for GAPDH was also increased in rIL-2–activated NK cells compared with fresh spleen cells, but the difference was less than eightfold (*B*).

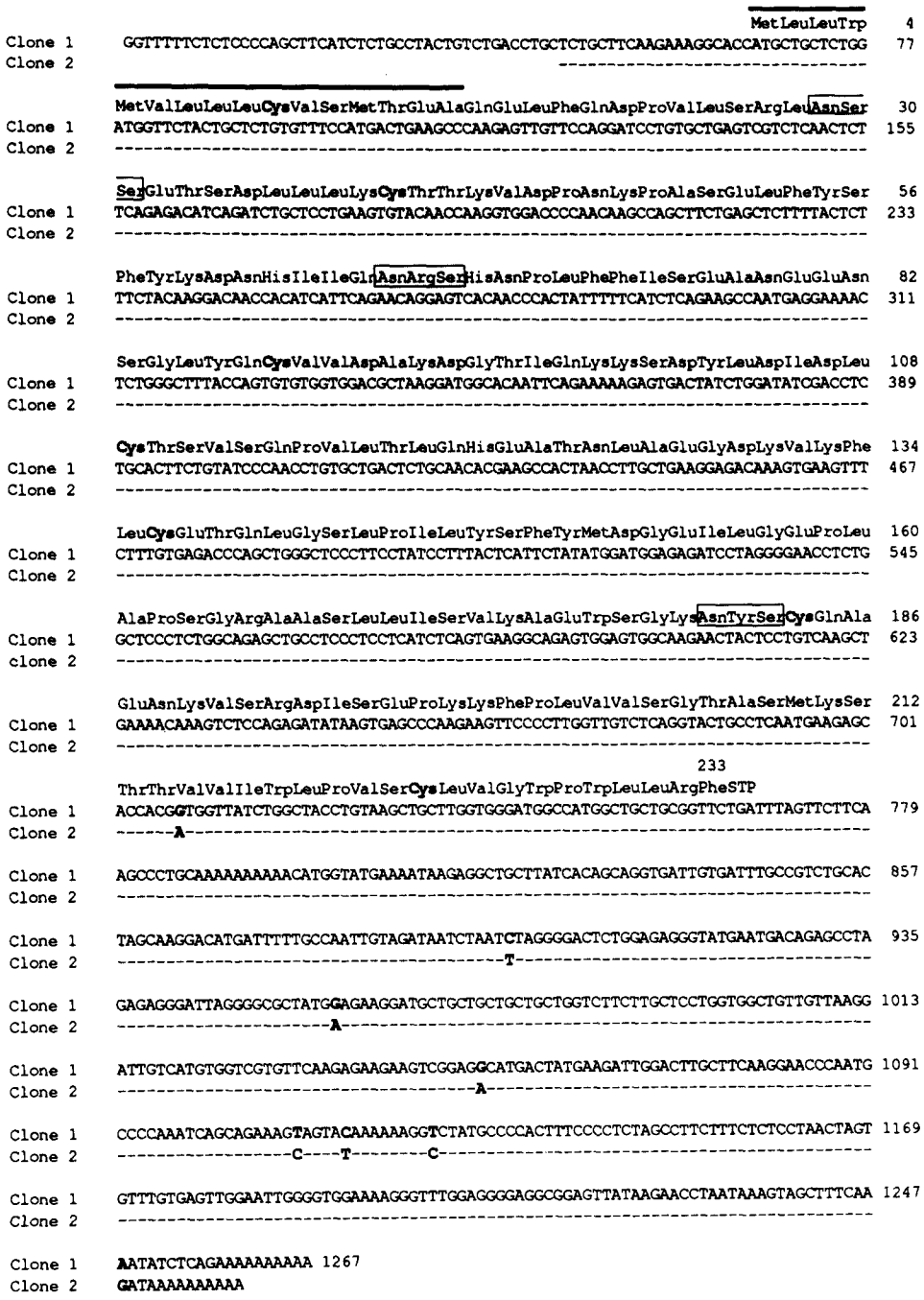

Figure 2. The nucleotide sequence of cDNAs for gp42 prepared from two independently derived clones. The sequence for clone 1 is given, together with the deduced amino acid sequence in the open reading frame. For clone 2, regions of nucleotide identity are indicated by a dashed line. The solid line overhead indicates the signal sequence, as defined by the criteria of von Heijne (29). Sites for N-linked glycosylation are bracketed. Cysteine residues are in boldface.

that were disparate between the two clones. The two clones contain an open reading frame with a deduced amino acid sequence of 233 residues (Fig. 2). The nucleotide sequences in the open reading frame are identical for the two clones, with the exception of a single nucleotide at position 708. This predicts a conservative change in the deduced amino acid sequence at position 214 (Met for Val). There are six additional nucleic acid differences between the two clones in the 3′ untranslated region. The differences could represent allelic products or mutations that arose during amplification of the cDNA. The two clones also differ in the site of attachment of the poly(A) tail, which begins 22 nucleotides from the AATAAA signal in clone 1 vs. 15 (or 16) nucleotides in clone 2 (Fig. 2).

Transformation of CHO cells with clone 2 led to high levels of expression of gp42, as detected by fluorescent staining with mAb 3G7 (see Fig. 4 *B*). This evidence that the clones contained a full-length open reading frame encoding gp42 was supported by the following: First, the translational start site is preceded by the nucleotide triplet ACC, in partial fulfillment of Kozak's criteria (28). Second, in the deduced amino acid sequence, the first 16 residues form a signal sequence, according

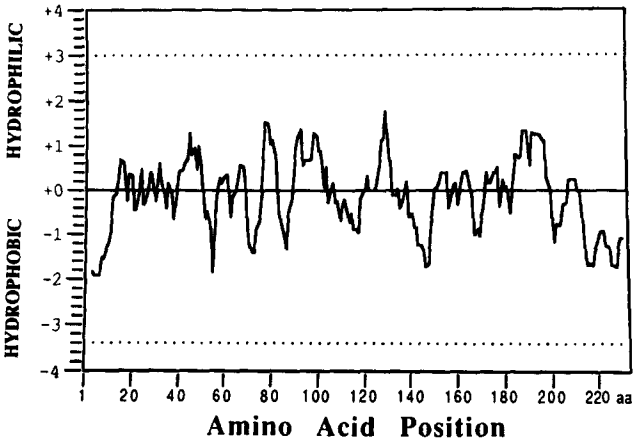

Figure 3. Hydrophilicity plot of the deduced amino acid sequence for gp42, demonstrating the hydrophobic signal sequence at amino acid positions 1–16, and the COOH-terminal hydrophobic region.

to the criteria of von Heijne (29) (Fig. 2, *dark line*). Third, the predicted molecular mass of the protein (26 kD) is similar to the observed molecular mass of deglycosylated gp42 (28 kD), and there are three potential sites for N-linked glycosylation (Asn-X-Thr/Ser; Fig. 2, *bracketed*). Further confirmation of the identity of the cDNA came from studies of the properties of gp42, as discussed below.

The Deduced Amino Acid Sequence for gp42 Predicts a Unique Protein that Is Attached to the Cell Membrane by a GPI Anchor. A search for homologies between the nucleotide sequence for gp42 and other vertebrate proteins was sought by a computer search of GenBank. No significant homologies were found; nucleotide homologies occurred with a normal distribution and did not extend beyond 3.6 SD in significance. Likewise, the deduced amino acid sequence for gp42 lacked extensive homology to any amino acid sequences in Swiss-Prot; the frequency of homologies followed a normal distribution and in only one instance did homology extend to five consecutive amino acids. In addition, the deduced amino acid sequence of gp42 was compared with all deduced sequences from GenBank. Again, no extensive homologies were found, but the highest match was of interest in that it was to the human high affinity Fc receptor, FcRI (CD64, reference 30), amino acid residues 172–374 (27% residue identity, significance 7.19 standard deviations above the mean score of 4,300 translated sequences compared). FcRI, FcRII (CD32), and FcRIII (CD16) show considerable homology to each other in the first two Ig-like domains, and this homology is conserved in rodents (30). The homology between gp42 and FcRI, however, began at the third extracellular domain of FcRI and extended through the transmembrane and cytoplasmic domains. An additional homology (GlyGluProLeuAla) was found between amino acids 157–161 of gp42 and 117–121 of FcRI. This is within the second Ig-like domain of FcRI, but the homology is not preserved in FcRII or FcRIII. The limited homologies between gp42 and FcRI may suggest a phylogenetic relationship between these two molecules, but gp42 appears to represent a molecule that has not previously been described in any species.

The plot of hydrophilicity for gp42 demonstrates the initial hydrophobic signal sequence and an additional hydrophobic region of ~16 amino acids at the COOH terminus (Fig. 3). The latter region lacks the length and composition of a transmembrane domain, and it is not followed by a cytoplasmic domain. Instead, a hydrophobic COOH terminus of 15–20 amino acids is one feature of glycoproteins that are attached to the cell surface by a GPI anchor (14, 15), suggesting this mechanism for attachment of gp42 to the cell membrane.

PI-PLC Releases gp42 from the Surface of RNK-16 Cells and from CHO Cells Transformed with the cDNA for gp42 but not from rIL-2–activated NK Cells. The gp42 molecule was first identified on the rat leukemia cell line RNK-16, which has features of NK cells (13, 31). Treatment of RNK-16 cells with PI-PLC reduced the level of gp42 on the cell surface by ~80% (Fig. 4 *A*), with no effect on the expression of transmembrane class I MHC molecules (not shown). Thus, most, if

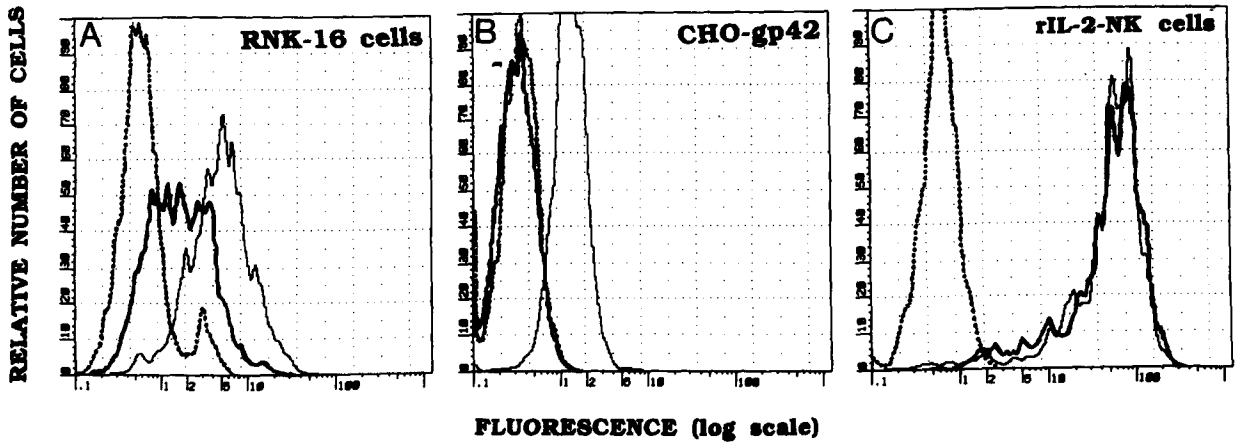

Figure 4. Effect of PI-PLC on the surface expression of gp42 on RNK-16 cells (*A*), CHO cells transformed with gp42 cDNA (*B*), and rIL-2–activated NK cells (*C*). (*Dotted line*) Background staining (isotype-matched fluorescein-conjugated mAb to the mouse antigen NK-1.1). (*Light line*) Staining of untreated cells with fluorescein-conjugated mAb to gp42 (3G7). (*Dark line*) Staining with the same anti-gp42 mAb after treatment with PI-PLC (0.5 U/ml for 1 h at 37°C).

not all, native gp42 on RNK-16 cells is attached to the cell membrane by a GPI anchor. PI-PLC also completely released gp42 from the surface of CHO cells that had been transfected with gp42 cDNA (Fig. 4 *B*). Treatment of rIL-2–activated NK cells with PI-PLC, however, did not release gp42 from the cell surface, even with higher concentrations of enzyme (up to 2 U/ml), as detected either by staining of the cells with fluorescein-conjugated antibody (Fig. 4 *C*) or by release of radiolabeled gp42, isolated by affinity chromatography with antibody 3G7 (not shown). This resistance to PI-PLC, however, was not unique to gp42; OX-45 is a GPI-anchored rat leukocyte antigen (18) that was largely released from fresh spleen cells by PI-PLC but that, when expressed on rIL-2–activated NK cells, was either not affected by PI-PLC or (in two of four experiments) was reduced only slightly (not shown). Thus, either gp42 and the OX-45 antigen are both expressed on rIL-2–activated NK cells in a form that is resistant to PI-PLC, or conditions on the surface of rIL-2-activated NK cells do not permit the activity of PI-PLC.

Studies by Presky et al., have demonstrated that GPI-anchored proteins on T lymphocytes become resistant to PI-PLC after activation by Con A, anti-CD3, or a combination of calcium ionophore and PMA (32). Our findings are consistent with a similar effect, in response to rIL-2, on GPI-anchored proteins expressed by NK cells. Alternatively, rIL-2 might stimulate the expression of a transmembrane form of gp42 by spleen cells, replacing the GPI-anchored form. To examine this point further, we first sequenced the 3′ end of the open-reading frame from two additional cDNA clones for gp42. Both were identical to clones 1 and 2. Thus, four independently derived clones from rIL-2–activated NK cells predict a GPI-anchored protein. Second, we used the PCR to amplify gp42 cDNA from rIL-2–activated NK cells, using primers from the 5′-translated and the 3′-untranslated ends of our cDNA. A single band was obtained which, when isolated and sequenced, encoded the GPI-anchoring terminus obtained from the λgt11 clones (not shown). Thus, either a message for a transmembrane form of gp42 is not present in these cells or the message is not homologous at its termini to the message for GPI-anchored protein. Finally, we immunoprecipitated surface-labeled gp42 from both rIL-2–activated NK cells and from RNK-16 cells and analyzed the degly-

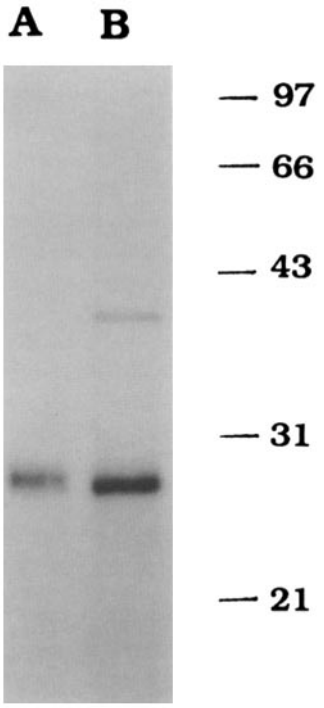

Figure 5. SDS-PAGE analysis of ^{125}I-labeled gp42 immunoprecipitated from rIL-2–activated NK cells (*A*) or from RNK-16 cells (*B*). This gel shows a size difference of ≤0.5 kD, the maximum difference that was observed on four analyses.

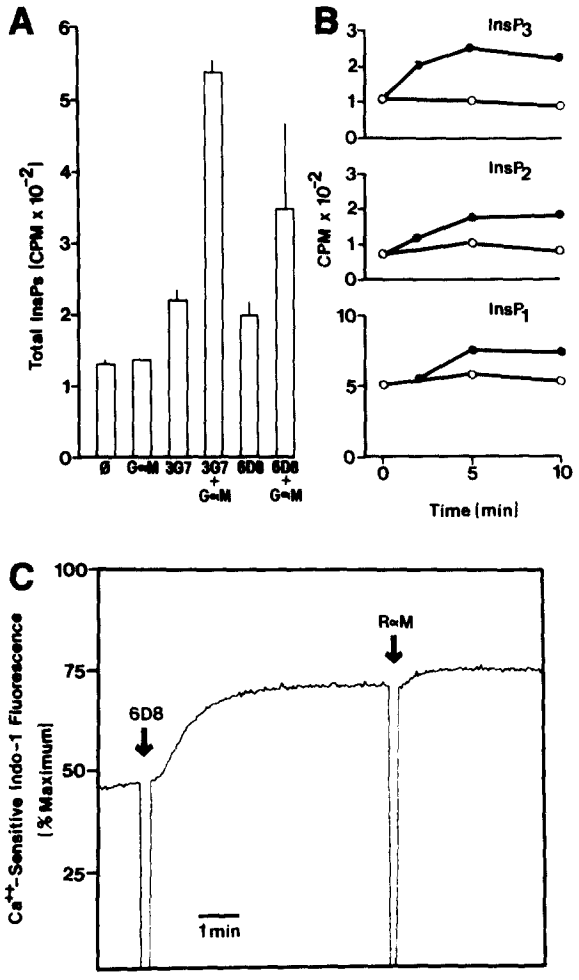

Figure 6. mAbs to gp42 stimulate increases in InsPs (*A*,*B*) and in $[Ca^{2+}]_i$ in RNK-16 cells. (*A*) Total InsPs after stimulation with either of two anti-gp42 mAb (3G7 or 6D8), alone or with a second-step goat antiserum to mouse Ig (GαM). [^{3}H]Inositol-labeled RNK-16 cells were incubated for 6 min with medium alone, with GαM alone, with anti-gp42 mAb alone, or with anti-gp42 followed 3 min later by GαM. The data represent the mean and range of duplicate samples. (*B*) Levels of $InsP_3$, $InsP_2$, and $InsP_1$, resolved by anion-exchange chromatography in RNK-16 cells after stimulation by 6D8 alone (*closed circles*), compared with medium alone (*open circles*). (*C*) $[Ca^{2+}]_i$ in RNK-16 cells, assessed by the Ca^{2+}-sensitive fluor Indo-1, in response to anti-gp42 (mAb 6D8) alone and with subsequent cross-linking by the addition of GαM. $[Ca^{2+}]_i$, calculated as described (20), was 185 nM in unstimulated cells and rose to 530 nM after the addition of 6D8 alone and to 660 nM after crosslinking with GαM.

cosylated protein by SDS-PAGE. A single band of ~28 kD was isolated from both. Analysis of four different gels indicated that the bands were either of identical size (not shown) or that gp42 from rIL-2–activated NK cells was only slightly (<0.5 kD) larger than gp42 from RNK-16 cells (Fig. 5). It is unlikely that this difference in size is sufficient to reflect the addition of a transmembrane region, especially if the molecule had any cytoplasmic domain. Although a small difference in size was seen in some gels, this may reflect modification of the GPI anchor, e.g., by acylation, which is associated with resistance to PI-PLC by GPI-anchored decay-accelerating factor (DAF) (33).

Perturbation of gp42 Causes an Increase in Phosphoinositides and in $[Ca^{2+}]_i$ in RNK-16 Leukemia Cells but not in rIL-2–activated NK Cells. Antibodies to certain GPI-anchored proteins on lymphocytes stimulate the production of soluble InsPs, and a consequent rise in $[Ca^{2+}]_i$, when the proteins are cross-linked on the cell surface (34–36). To examine the effect of gp42 mAb on the generation of InsPs, RNK-16 cells were incubated with [^{3}H]inositol and then exposed to anti-gp42. Alone, the mAb stimulated an ~50% increase in total [^{3}H] InsPs (Fig. 6 *A*), and a two- to threefold increase in [^{3}H]inositol trisphosphate ($InsP_3$, Fig. 6 *B*). The rise in total InsPs was augmented when anti-gp42 was cross-linked by a second-step antiserum to mouse Ig (Fig. 6 *A*). Consistent with the ability of $InsP_3$ to mobilize intracellular Ca^{2+}, anti-gp42 mAb alone stimulated a substantial rise in $[Ca^{2+}]_i$ that was further augmented by cross-linking (Fig. 6 *C*).

RNK-16 is cytotoxic to tumors that are killed by fresh rat NK cells, and we have previously shown that RNK-16 cells respond to susceptible targets by generating a rise in InsPs (20). In our previous studies, a rise in InsPs was also generated by cross-linking anti-CD2 mAb (OX-34) on the surface of RNK-16 cells (20). Anti-CD2 alone did not stimulate a rise in InsPs, but it blocked the response of RNK-16 cells to target cells, as assessed both by cytotoxicity and by changes in InsPs (20). Although cross-linking anti-gp42 also generated a rise in InsPs and in $[Ca^{2+}]_i$ within RNK-16 cells, soluble anti-gp42 alone did not block the response of RNK-16 cells to targets as assessed either by cytotoxicity (13) or by changes in InsPs (not shown). Also, despite the abilities of gp42 and CD2 to trigger increases in $[Ca^{2+}]_i$ and in InsPs within RNK-16 cells, these responses could not be elicited in rIL-2–activated NK cells by cross-linking either anti-gp42 or anti-CD2 (not shown).

Discussion

We have cloned the cDNA for rat gp42, a cell surface glycoprotein that is selectively induced on NK cells in response to rIL-2, and we have expressed the gene in CHO cells. Neither the nucleotide nor the amino acid sequence of gp42 demonstrates significant homology to other known proteins. Several properties of the gp42 molecule are of interest.

First, the deduced amino acid sequence predicts a molecule that is attached to the cell membrane by a GPI anchor. In accord with this, treatment with PI-PLC markedly reduced the expression of gp42 on RNK-16 cells, which constitutively express gp42, or on CHO cells that were transformed with the cDNA for gp42. In contrast, treatment of rIL-2–activated NK cells with PI-PLC had no effect on the expression of gp42. Thus, either gp42 is expressed in a different manner on rIL-2–activated NK cells, or conditions on these cells do not permit the activity of PI-PLC. With regard to the first possibility, there are several surface molecules that can be expressed either as GPI-anchored or as transmembrane structures. An example is the immunoglobulin receptor, CD16, which is GPI-anchored on polymorphonuclear cells but expressed as a transmembrane glycoprotein on NK cells (37, 38). Thus, it is possible that gp42 might be expressed on rIL-2–activated NK cells as a transmembrane protein. Our studies, however, argue against this. First, our cDNA clones were prepared from mRNA that was obtained from rIL-2–activated NK cells, and four independently derived clones lack an apparent transmembrane domain. Second, Northern blot analysis of rIL-2–activated NK cells with gp42 could detect only a single message (Fig. 1), making it unlikely that these cells express more than one form of gp42. Third, a single cDNA species was obtained by PCR from mRNA prepared from rIL-2–activated NK cells. This argues against IL-2–induced expression of a transmembrane form that is highly homologous to the GPI form, as is the case for different forms of CD16 (38). Fourth, immunoprecipitation of surface-labeled gp42 from rIL-2–activated NK cells revealed a single structure that was nearly identical in size to gp42 on RNK-16 cells. Finally, PI-PLC treatment was relatively ineffective in cleaving from rIL-2–activated NK cells not only gp42 but also the antigen OX-45, which is expressed on freshly obtained cells as a GPI-anchored protein (18). In all, these findings suggest that gp42 on rIL-2–activated NK cells is attached by a GPI anchor, as it is on RNK-16 cells, but it is not readily cleaved by PI-PLC. To further exclude the alternate possibility that gp42 could exist as a transmembrane protein, we have performed Southern blot analysis of rat genomic DNA using gp42 cDNA. This identifies an EcoRI fragment of ~13 kb. Partial mapping and sequencing of a genomic clone confirm that there is a 13-kb EcoRI fragment that encodes a single gene for GPI-anchored gp42, including all but the 5′-untranslated region and the start of the signal sequence (unpublished studies). There is weaker and more variable hybridization to fragments of ~8 kb and ~3 kb, so it is possible that there are additional genes present with at least partial homology to gp42.

gp42 was initially identified on the rat leukemia cell line RNK-16. This cell line has features of NK cells (30), but gp42 is not expressed in detectable amounts on freshly prepared rat leukocytes, including NK cells (13). Instead it is uniquely induced on NK cells in response to IL-2 (13). Fresh NK cells express the p70 component of the IL-2 receptor at much higher levels than other lymphocytes, and they may thus respond more promptly to stimulation by IL-2 (4). However, gp42 is not detectable on lymphocytes other than NK cells even after 6 d of culture in IL-2 (13). The unique expression of gp42 on rIL-2–activated NK cells, therefore, is not simply the consequence of a more ready response, but rather a lineage-specific response.

The selective expression of gp42 suggests a possible role for this molecule in regulating responses that are unique to rIL-2–activated NK cells. The physiologic function of gp42, however, has not been determined. The study of other GPI-linked surface proteins on mammalian cells indicates that certain of these, such as LFA-3 and N-CAM, are adhesion molecules (39–41). Others, including alkaline phosphatase (42), acetylcholinesterase (43), and 5′-nucleotidase (44) have enzymatic activity. Of particular relevance to gp42 may be the demonstration that T lymphocytes can be activated when certain GPI-anchored proteins are cross-linked in the presence of PMA. These include certain Ly-6 isoforms (45), Thy-1 (46), DAF (47), and Qa-2 (48), all of which are expressed on T cells as GPI-anchored proteins (49–53). The T cell proliferative responses to anti-Thy-1 and to anti-Ly-6 have been studied in greatest detail. Both responses are dependent on the expression of the TCR/CD3 complex; T cell lines lacking the TCR/CD3 complex are not activated, and the response is restored when expression of the complex is restored (54, 55). For Ly-6, the opposite has also been demonstrated; inhibition of Ly-6 expression by antisense oligonucleotides prevents activation of T cells through the TCR (56). Thus, GPI-anchored proteins may play a vital role in lymphocyte activation. The activation of T cells through the TCR/CD3 complex involves the generation of InsPs with a consequent rise in $[Ca^{2+}]_i$ (57). Cross-linking of either Thy-1 or Ly-6 also stimulates a rise in $[Ca^{2+}]_i$, suggesting that this response might be involved in lymphocyte activation through Thy-1 or Ly-6 (34, 35).

We found that gp42 stimulates the generation of InsPs and a rise in $[Ca^{2+}]_i$ in RNK-16 leukemia cells. Because this is a property of other GPI-linked molecules that activate lymphocytes, it is possible that gp42, when it is expressed, might similarly be involved in providing activation signals to lymphocytes. We could not, however, detect changes in InsPs or in $[Ca^{2+}]_i$ when gp42 was cross-linked on the surface of rIL-2–activated NK cells. These responses were also not obtained in rIL-2–activated NK cells in response to cross-linking CD2 or exposing cells to Con A or to YAC-1 target cells (unpublished observations). These findings are similar to those of Zanovello et al., who found that mouse lymphokine-activated killer (LAK) cells do not generate InsPs or a rise in $[Ca^{2+}]_i$ in response to target cells (58). They also found that murine LAK cells can kill targets in the absence of extracellular Ca^{2+}, indicating that murine LAK cells may use other activation pathways to generate cytotoxic activity (58). Stahls and Carpen, however, found that culture of human NK cells in rIL-2 increased the hydrolysis of phosphatidylinositols in response to target cells (59). Our previous studies with gp42 indicate that it is not required for the generation of cytotoxicity by rIL-2–activated NK cells (20), but the effect of gp42 on other activation events in NK cells has not yet been investigated.

Regardless of the function of gp42, it provides a highly specific marker for rIL-2–activated NK cells that will be useful for in vivo studies of their migration and activity. The selective induction of gp42 on NK cells in response to IL-2 provides a model for studying the cell-specific response to this cytokine. The isolation of the cDNA for gp42 will enhance functional studies of the molecule, will permit isolation of the genomic DNA to define the basis of its lineage specific response to IL-2, and may permit the isolation of homologous molecules from other species, including humans.

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