# NKR-P1, a Signal Transduction Molecule on Natural Killer Cells

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**Natural killer (NK) cells are a subpopulation of large granular lymphocytes characterized by densely staining azurophilic granules. NK cells are able to recognize and lyse various virally infected or neoplastic target cells without previous sensitization or major histocompatibility complex restriction. A 60-kD disulfide-linked dimer, highly expressed on NK cells, was found capable of mediating transmembrane signaling. The gene encoding this signal transduction molecule was cloned and its nucleotide sequence determined. The encoded protein showed significant homology with a number of lectin-related membrane proteins that share receptor characteristics. This protein may function as a receptor able to selectively trigger NK cell activity.**

THE NATURE OF THE SIGNAL TRANSduction molecules specifically involved in target cell recognition by NK cells (the putative NK receptor) has so far eluded biochemical identification (*1*). NK cells do not rearrange T cell receptor (TCR) genes (*2*) and do not express cell surface TCR molecules or components of the cluster-of-differentiation 3 (CD3) transduction complex (*3*). Most NK cells do express CD2 and CD16 signal transduction molecules (*4–6*), which are able to stimulate internal $Ca^{2+}$ mobilization as well as enhance NK cell–mediated killing of appropriate Fc receptor–positive ($FcR^+$) cells, that are otherwise NK-resistant targets (for example, P815) (*7, 8*). However, monoclonal antibodies (MAbs) directed against these two molecules fail to inhibit recognition and lysis of NK-susceptible target cells (for example, YAC-1) (*8*). Further, $CD2^-$ and $CD16^-$ NK cells can also be isolated, and both express high levels of lytic activity (*9*). This would suggest that although these molecules may participate in signal transduction, neither of them are required for NK activity. Monoclonal antibodies specific for another triggering structure, CD45, have been reported to inhibit NK cell lysis of selected tumor cells, suggesting a possible role in conferring NK cell specificity (*5, 6, 10*). However, CD45 is not NK cell–specific, as it is expressed on virtually all hematopoietic cells except mature erythrocytes and their immediate precursors (*6*).

We recently isolated a mouse immunoglobulin G1κ (IgG1κ) MAb, 3.2.3, which identified a structure expressed at high density on rat NK cells. The antibody induced enhanced killing against appropriate $FcR^+$, NK-resistant target cells and, when cross-linked, also stimulated NK cells to secrete butoxycarbonyl-lysyl-tyrosine-esterase, an enzyme commonly associated with exocytosis of cytolytic granules (*11*). MAb 3.2.3 precipitated a structure of 60 kD, which, under reducing conditions, migrated as a single 30-kD band and suggested a dimeric structure for the 3.2.3 antigen (*11*). We sought to isolate the gene encoding the 3.2.3 antigen in order to better understand its role in NK cell–specific function.

Because of the dimeric structure and the possible glycosylated nature of the 3.2.3 antigen, it was necessary to clone the gene from a library expressed in eukaryotic cells. We created a cDNA library with mRNA from highly purified, interleukin-2 (IL-2)–stimulated, rat NK cells (*12*), and inserted it into pCMD8, a vector that is able to mediate an over expression of the inserted genes in particular cell lines such as COS-7 (*13*). Transiently transfected COS-7 cells were enriched for the insert of interest by "panning" (*13*). The cells that were recognized by the 3.2.3 MAb were anchored to plastic petri dish surfaces coated with goat antibodies specific for mouse IgG (*14*). After three rounds of panning, about 90% of the recovered plasmids had inserts of very similar size (~1.1 kb) that cross-hybridized strongly with each other. Ten percent had heterogeneous inserts that did not. Sequencing (*15*) of two clones with the largest size difference

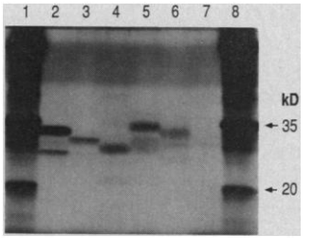


**Fig. 2.** Determination of protein orientation in the cell membrane. For cotranslational processing, mRNA was prepared by transcription, with T7 polymerase, of the Not I–digested plasmid containing the 3.2.3 cDNA. After purification by phenol-chloroform extraction and ethanol precipitation, the mRNA was translated with rabbit reticulocyte lysate (Promega) in the presence of [$^{35}$S]cysteine according to the supplier's protocol. Canine microsomes (Promega) were present during the translations of samples in lanes 3, 5, 6, and 7. The 3.2.3 cotranslationally processed sample was further analyzed by proteinase K (Sigma) digestion as described (*22*). All samples were separated by electrophoresis in a 15% polyacrylamide gel. Lanes 1 and 8 contain the products from Brome Mosaic Virus mRNA as a translation control (20- and 35-kD bands are indicated). Lanes 2 and 3 contain the product from *Escherichia coli* β-lactamase mRNA, and lanes 4 to 7, the product from 3.2.3 mRNA. Before electrophoresis, sample 7 was supplemented with Triton X-100 and both samples 6 and 7 were then treated with proteinase K. The size reduction of the β-lactamase from 31.5 to 28.9 kD demonstrates the efficient signal processing activity of the microsomes. The increased size of the 3.2.3 product (lane 5 versus 4, 34 and 26 kD, respectively) reveals its in vitro glycosylation, whereas the slight size reduction (lane 6 versus 5) indicates that the microsomal vesicle restricts the access of the protease to only a small portion of the molecule. Both observations suggest that the larger carboxyl-terminal domain was inside the microsomal vesicle and correspondingly it is normally located on the outside of the cell membrane.

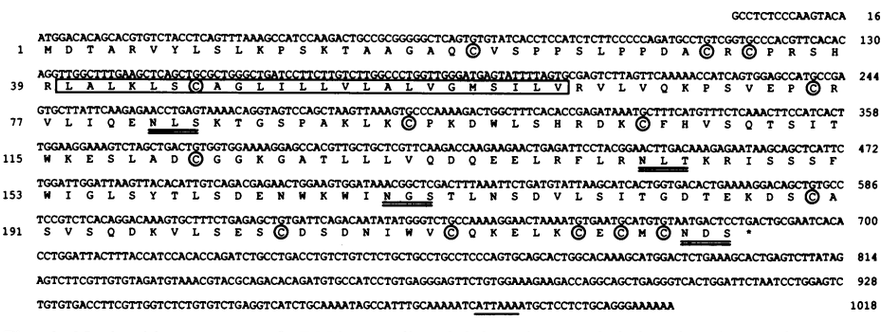


**Fig. 1.** Nucleotide sequence of cDNA encoding 3.2.3 antigen and deduced amino acid sequence. Nucleotides are numbered on the right side, amino acids on the left side (*21*). The cysteine residues are circled and the transmembrane portion is boxed. Potential N-linked glycosylation sites are doubly underlined. A possible, although atypical, polyadenylation consensus sequence is underlined.

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(about 30 bases), showed that they had identical sequences, except that the shorter clone had 7 bases less at the 5′ end and a slightly shorter polyadenylated tail. The sequence of the 1018-bp cDNA encoding the 3.2.3 antigen is presented in Fig. 1. A single open reading frame codes for a peptide of 223 amino acids with a calculated molecular mass of 24,551 daltons. This protein contains a clearly defined hydrophobic portion (from residue 40 to 63), with the characteristics of a membrane-spanning region, separating a short amino-terminal part from the longer carboxyl-terminal portion that contains a number of potential glycosylation sites. Glycosylation at these sites could explain the difference between the calculated molecular mass and that observed after immunoprecipitation (24.5 and 30 kD, respectively). The orientation of the 3.2.3 protein in the cell membrane was determined by cotranslational processing (Fig. 2). The results indicated that the protein was oriented with the carboxyl-terminal portion outside of the cell membrane and that it is thus a type II transmembrane protein (*16*). Amino acid sequence comparisons revealed a significant similarity with the lectin motif present in a number of other proteins (Fig. 3). A similar secondary structure for this group of molecules that share receptor characteristics is suggested by conservation of the number and positions of several cysteines. In addition, the presence of serine residues in the cytoplasmic portion of the molecule (potential phosphorylation sites) is consistent with a signaling function for this protein.

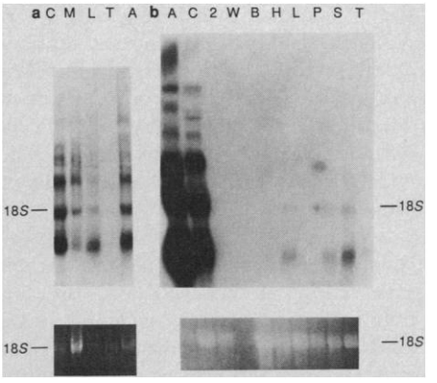


**Fig. 5.** (**a**) Northern blot analysis of total RNA from different cell subpopulations. RNA extraction, electrophoresis, transfer, and hybridization were as in (*17*). RNA (20 μg per lane) was separated by electrophoresis in a gel containing 1.5% agarose and 3% formaldehyde. Fragments to be used as hybridization probes were isolated in low melting point agarose (Bethesda Research Laboratories), and labeled to a specific activity of $10^9$ cpm/μg with Multiprime (Amersham). The 3.2.3 probe hybridizes to an ~1.1-kb band on rat A-LAK (lane A) and CRNK-16 (lane C) mRNA. Polymorphonuclear lymphocytes (lane M), peripheral blood lymphocytes (lane L), and T cells (lane T) show decreasing quantities of 3.2.3-specific mRNA. Bands of higher molecular weight may be due to partially spliced forms of the mRNA. (**b**) Analysis of the total RNA extracted from fresh whole rat organs. Expression of 3.2.3 message is evident in the spleen (lane S), and, in decreasing amounts, in heart, lungs, and thymus (lanes H, P, and T, respectively). Whole blood (lane W), liver (lane L), and brain (lane B) were negative. The yolk sac tumor cell line L2 (lane 2) and CRNK-16 (lane C) RNAs were added as negative and positive controls, respectively. In lane A, we loaded a minimal quantity of polyadenylated mRNA from A-LAK cells as 3.2.3 molecular weight marker. Below each autoradiogram is the ethidium bromide–stained 18*S* ribosomal RNA region of the gel to indicate that similar quantities of total RNA were loaded in each lane. Although an insufficient amount of RNA was loaded in lane W of the gel in (b), other experiments confirmed the very limited amount of specific mRNA from whole blood (*18*).

To confirm that this gene actually encodes the antigen recognized by the 3.2.3 MAb, we performed transfection experiments with the cloned cDNA (Fig. 4). The 3.2.3-specific message (~1.1 kb) was found in large quantities in RNAs from both NK cells and a tumor cell line with NK characteristics, CRNK-16 (*11*), but was absent in T cells and other cell lines of rat origin that do not express NK activity [for example, L2 cell line (*17*)] (Fig. 5a). Northern analysis of the total RNA extracted from fresh whole rat organs showed the presence of 3.2.3-specific message in the spleen, and in very limited and decreasing amounts in heart, lungs, thymus, liver, whole blood, and brain (Fig. 5b). These findings are entirely consistent with the distribution of NK cells and MAb 3.2.3 reactivity.

Southern blot analysis of rat genomic DNA, treated with restriction enzymes that do not cut within the cDNA sequence, revealed from two to seven fragments that hybridized at high stringency with the cDNA used as radiolabeled probe. When portions of this cDNA were used as probes, each hybridized to a smaller subset of the same bands (*18*). This can be considered a

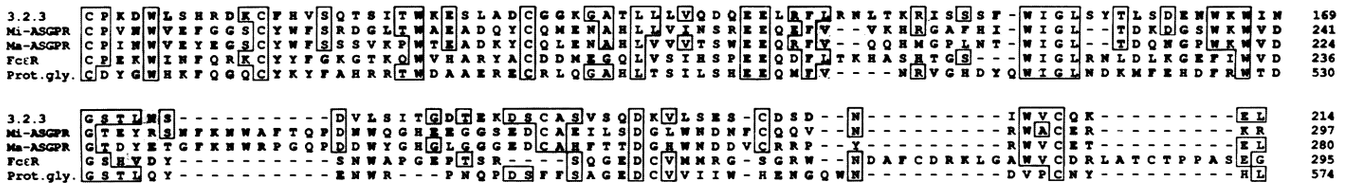


**Fig. 3.** Optimal alignment of amino acids 94 to 214 of the 3.2.3 protein with the lectin-like portions of minor rat asialoglycoprotein receptor (Mi-ASGPR) (*23*), major rat asialoglycoprotein receptor (Ma-ASGPR) (*24*), human low-affinity IgE receptor (FcεR) (*25*), fibroblast chondroitin sulfate proteoglycan core protein (Prot.gly.) (*26*). Amino acids that are identical to those of 3.2.3 are boxed. Conservative substitutions are shaded. Spaces introduced to optimize alignment are indicated by dashes.

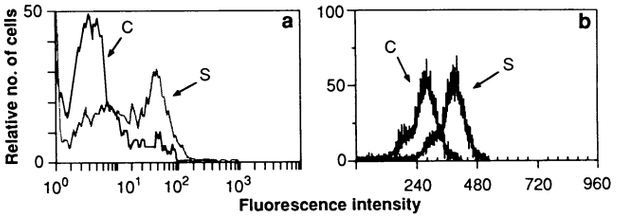


**Fig. 4.** Expression of the molecule identified by MAb 3.2.3 on COS-7 and PA317 cells after transient and permanent transfections, respectively. (**a**) Flow cytometric (FACStar, Becton-Dickinson) analysis of nontransfected, 3.2.3$^-$ COS-7 cells treated with MAb 3.2.3 revealed by fluorescent goat antibodies to mouse (profile C). Superimposed is the profile of COS-7 cells transfected, by Lipofectin (Bethesda Research Laboratories), with 3.2.3-specific cDNA inserted in plasmid CDM8 and otherwise treated exactly like the previous ones (profile S). When these cells were treated instead with a nonspecific mouse immunoglobulin of the same subclass as 3.2.3 MAb, the profile was similar to that of profile C. (**b**) Flow cytometry (FACStar-Plus) analysis of PA317 mouse fibroblasts transfected by Lipofectin with 3.2.3-specific cDNA inserted into vector RC/CMV. Fluorescent F(ab′)$_2$ fragments of MAb 3.2.3 were used to reveal that the 3.2.3 antigen was not expressed on a cell clone transfected with vector RC/CMV, which did not contain the 3.2.3 cDNA (profile C). These results are superimposed with those from a 3.2.3 cDNA–positive PA317 clone treated with the same F(ab′)$_2$ reagent (profile S). The 3.2.3$^+$ transfected cells showed an overlapping profile with the one shown in profile C when treated with fluorescent F(ab′)$_2$ fragments from an unrelated antibody of the same subclass as 3.2.3 MAb.

strong indication that there is only one rat 3.2.3 gene and that this gene may consist of at least seven exons. Further, genomic DNA analysis from either $3.2.3^+$ or $3.2.3^-$ rat cell lines showed the same pattern of fragments (*18*), demonstrating that the gene does not undergo rearrangement in order to be expressed.

All these data indicate that the gene encoding the 3.2.3 antigen is not related to the genes encoding other NK cell–associated transduction molecules, including CD2, CD16, and CD45. The membrane orientation and the similarities of the deduced protein sequence to the sequences of the receptor molecules shown in Fig. 3 qualify our NK cell protein as a member of the C-type animal lectin family (*19*). This similarity to the C-type lectins implicates a calcium-dependent ligand binding process and also suggests that the ligand is a carbohydrate because many of these lectins specifically bind oligosaccharides with terminal galactose, *N*-acetylglucosamine, or mannose. However, the possibility that the NK cell protein could bind specific peptides is not excluded, because such binding has been demonstrated in the case of the low-affinity IgE receptor (FcεR) (*20*). Finally, the lectin-like domain of the NK cell protein has twice as many lysine residues as the majority of the C-type lectins. This suggests that the receptor ligand may be more negatively charged than the ligands of the other C-type lectins.

One could speculate that the NK protein also shares functional characteristics with the most similar C-type lectins. As an example, the low-affinity IgE receptor has been demonstrated to be involved in B cell activation (*20*). The participation of our protein in activation of NK cells is suggested by its structural characteristics and is also implied by the effects of the 3.2.3 antibody on NK function.

We conclude that our protein (NKR-P1) functions as a receptor expressed selectively on NK cells, which shares similarities with molecules whose surface expression is linked to cell activation programs.


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14. Total cellular RNA was extracted from $10^8$ rat adherent IL-2–activated killer (A-LAK) (*12*) cells, with the use of a modification of the guanidine isothiocyanate method (*17*). Polyadenylated [poly(A)$^+$] RNA was purified on an oligo(dT) cellulose column (Bethesda Research Laboratories); cDNA was synthesized from 3 μg of poly(A)$^+$ RNA by means of a cDNA synthesis kit (Amersham). Bst XI linkers were added to the cDNA, and a library of $1.2 \times 10^6$ primary clones was made in Bst XI–cut CMD8 plasmid (*13*), and amplified in *Escherichia coli* MC1061/P3 cells (*13*) (Invitrogen). COS-7 cells at 50% confluency were transfected by Lipofectin (Bethesda Research Laboratories) with CsCl-purified DNA from the amplified library. Panning was performed as described (*13*), with MAb 3.2.3. from ascites fluid as first antibody, at a dilution of 1:1,000, and as second antibody, goat antibody to mouse IgG (Sigma).
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27. We thank R. B. Herberman, E. Turco, P. Basse, and C. A. Dahl for their suggestions and technical advice; C. Robinson and R. Fritsch for technical assistance; and J. P. McCoy for cell sorting. Partially supported by National Cancer Institute grant CA 44977, National Institute of Allergy and Immunology grants AI 23963 and AI 26364, and a grant from the Wendy Will Case Cancer Fund, Inc.



6 March 1990; accepted 23 July 1990


# Expression and Activity of the POU Transcription Factor SCIP


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**POU proteins have been shown to transcriptionally activate cell-specific genes and to participate in the determination of cell fate. It is therefore thought that these proteins function in development through the stable activation of genes that define specific developmental pathways. Evidence is provided here for an alternative mode of action. The primary structure of SCIP, a POU protein expressed by developing Schwann cells of the peripheral nervous system, was deduced and SCIP activity was studied. Both in normal development and in response to nerve transection, SCIP expression was transiently activated only during the period of rapid cell division that separates the premyelinating and myelinating phases of Schwann cell differentiation. In cotransfection assays, SCIP acted as a transcriptional repressor of myelin-specific genes.**


THE DEVELOPMENT OF MYELIN-forming Schwann cells, the principal glial cells of the vertebrate peripheral nervous system, can be divided into premyelinating and myelinating phases (*1*). These phases are defined by the reciprocal expression of marker genes (*2, 3*) and are separated by a transition period during which Schwann cells rapidly divide (*4*). Both the initial progression of Schwann cells to the myelinating phase, as well as the subsequent maintenance of this phenotype, depend on a contact-mediated interaction with axons (*5, 6*). In cultured Schwann cells, early features of this interaction, including the triggering of cell division and the partial induction of myelin-specific genes, can be induced by agents that elevate intracellular concentrations of adenosine 3′,5′-monophosphate (cAMP) (*7, 8*).

We asked whether a POU protein might be involved in mediating Schwann cell differentiation. We used degenerate oligonucleotides corresponding to the homeobox


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