ALTERNATIVE MEMBRANE FORMS OF FcγRIII(CD16) ON HUMAN NATURAL KILLER CELLS AND NEUTROPHILS

Cell Type-Specific Expression of Two Genes That Differ in Single Nucleotide Substitutions

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Receptors for the Fc fragment of IgG (FcγR) couple the humoral and cellular immune responses by targeting immune complexes to effector cells. Multiple FcγRs exist, which differ in ligand affinity, cellular distribution, and effector function (reviewed in reference 1). Detailed characterization of the FcγRs in both mouse and human has begun to address the molecular basis for the diversity of cellular responses triggered by a common ligand. The binding of immune complexes is mediated by extracellular Ig-like domains that are conserved among many FcγRs. The functional consequence of this binding, on the other hand, is mediated by the divergent transmembrane and cytoplasmic domains that are the result of gene duplication as well as alternative mRNA splicing. In the mouse the low affinity, immune complex IgG Fc receptors (FcγRII) are encoded by two genes, α and β (2–4). cDNA sequence analysis predicts that these receptors are similar integral membrane glycoproteins with 180 amino acid extracellular domains, single transmembrane spanning domains of 20 amino acids, and intracytoplasmic domains that vary from 26 amino acids for α to 93 amino acids for the larger spliced form of β, β_1. α is expressed on macrophages and NK cells, while β is expressed on lymphocytes and macrophages and displays cell type–specific alternative mRNA splicing of its cytoplasmic domains. The human homologues of these receptors include a minimum of three genes for FcγRII(CD32) (Qiu, W. Q., and J. Ravetch, unpublished observations) and two genes for FcγRIII(CD16) (this article). cDNA clones have been isolated for FcγRII(CD32) (5, 6; Brooks, D., W. Q. Qiu, A. Luster, and J. Ravetch, manuscript submitted for publication) and FcγRIII(CD16) (7, 8).

FcγRIII(CD16) is expressed on NK cells, macrophages, and PMN (9–11). Two alleles, NA-1 and NA-2, have been described for this receptor on PMN. It has been shown to mediate antibody-dependent cellular cytotoxicity (ADCC)[1] by NK cells

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; PI-PLC, phosphatidyl inositol–specific phospholipase C; PNH, paroxysmal nocturnal hemoglobinuria.

(12), where it represents the only FcγR. On PMN, FcγRIII(CD16) has been proposed to act together with FcγRII(CD32) to mediate effector functions (13, 14). Anti-FcγRIII(CD16) antibodies inhibit ADCC and immune complex binding (9, 10, 15) on both PMN and NK cells. Recently it has been shown that FcγRIII(CD16) is anchored through a glycosyl-phosphatidylinositol (PI) linkage on PMN (13, 16). The first evidence for an alternative membrane-associated form for FcγRIII(CD16) came from the study of patients with paroxysmal nocturnal hemoglobinuria (PNH). In that acquired disorder, a defect in the attachment of the PI tail in hematopoietic precursor cells results in the selective deficiency of PI-anchored proteins (reviewed in reference 17). FcγRIII(CD16) is expressed at 10% of normal levels on PMN in those patients, but its expression on macrophages and NK cells is unaffected (16, 18), indicating that NK cells express an alternative anchored form of FcγRIII(CD16) that is presumably transmembrane. cDNA clones for FcγRIII(CD16) have been isolated from placental and neutrophil libraries (7, 8). Those clones predict a sequence for an FcγRIII(CD16) protein that contains two canonical Ig-like extracellular domains, a weakly hydrophobic transmembrane domain and a short (four amino acid) cytoplasmic domain, features characteristic of PI-linked molecules (17, 19). Transfection of those clones resulted in the appearance of PI-linked molecules on COS cells; thus, it appeared unlikely that these cDNA clones encoded a transmembrane form of FcγRIII(CD16).

In this report we demonstrate that the FcγRIII(CD16) molecule indeed exists in two alternative membrane-anchored forms, a PI-linked form on PMN and a larger PI-PLC–resistant transmembrane protein on NK cells. To establish the molecular basis for this difference, FcγRIII(CD16) encoding RNA from NK cells and PMN of single individuals homozygous for either NA-1 or NA-2 were analyzed and found to differ by multiple single nucleotide substitutions. One of these nonallelic changes results in the expression of a transcript in NK cells in which a CGA codon replaces a UGA termination codon thereby extending the reading frame for the cytoplasmic domain of this FcγRIII(CD16) by 21 amino acids, which are homologous to the murine FcγRIIα cytoplasmic domain. Two distinct genes encoding FcγRIII(CD16) have been cloned and sequenced. Cell type–specific expression of these linked genes accounts for the NK cell and PMN transcripts and the alternatively anchored forms of this receptor.

Materials and Methods

Cell Lines.   The human B lymphoblastoid cell line RPMI 8866, and the mAb-producing hybrid cell clones were maintained in culture in RPMI 1640 (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% FCS (Flow Laboratories, Alexandria, VA). All cell lines were free of mycoplasma contamination.

Monoclonal and Polyclonal Antibodies.   The mAbs used in this study, their origin, and specificity have been previously described (10, 20). Anti-CD16 mAbs were: B73.1 (IgG1) produced and characterized in our laboratory (10), 3G8 (9) (IgG1) produced from cells kindly provided by Dr. J. Unkeless (Mount Sinai Medical School, New York, NY) and CLB-Gran 11 (21), detecting the NA-1 alloantigen on PMN and GRM1, detecting the NA-2 antigen (kindly provided by F. Garrido). TS2/9 (anti-LFA-3) (22) was kindly provided by T. Springer (Harvard Medical School, Boston, MA). IgG were purified from ascites and labeled with biotin according to routine procedures. The polyclonal FITC-labeled goat F(ab')₂ anti–mouse Ig was purchased from CooperBiomedical Inc. (Malvern, PA). The goat anti–mouse IgG used to prepare erythrocytes (E) for indirect rosetting was produced in our laboratory, absorbed

on human IgG, and affinity purified on mouse IgG-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden).

Peripheral Blood Leukocytes, NK Cells, Polymorphonuclear Granulocyte (PMN) and Macrophage Preparations. Venous peripheral blood was obtained from adult healthy donors and anticoagulated with heparin. Buffy coats, PMN, PBMC, lymphocytes (PBL), and the NK cell subset were obtained as previously described in detail (10, 15). Monocytes were prepared from PBMC by adherence to plastic (45 min, 37°C) and depleted of contaminating lymphocytes by complement (C)-dependent lysis (45 min, 37°C) after treatment with a mixture of C-fixing antibodies anti-CD21, anti-CD16, and anti-CD3. Macrophages were collected after culturing this population for 10–12 d in RPMI-1640 supplemented with 10% human serum. Both NA-1 and NA-2 homozygous donors were used. To obtain large numbers of homogeneous CD3$^-$/CD16$^+$ NK cells, PBMC were cocultured with 50 Gy-irradiated RPMI 8866 cells, as described (20). These 10-d cultures contain, on average, 80% CD3$^-$/CD16$^+$/NKH-1$^+$ NK cells and 20% CD3$^+$/CD16$^-$/NKH-1$^-$ T lymphocytes. The NK cells were purified by negative selection using antiglobulin rosetting and density gradient centrifugation after sensitization of the lymphocytes with a mixture of anti-CD3, anti-CD5, and anti-CD14 mAbs. These NK cell populations have morphologic, phenotypic, and functional properties identical to those of NK cells freshly purified from blood (20, 23). Like these cells, in vitro propagated NK cells express functional FcγRIII(CD16), but neither FcγRII(CD32) nor the high affinity receptor for the Fc fragment of monomeric IgG, FcγRI. The purity of each leukocyte preparation was tested by indirect immunofluorescence (flow cytometry) using anti-NK (anti-CD16 and anti-NKH-1), anti-T (anti-CD3 and anti-CD5), antimonocyte (anti-CD14) and anti-PMN (anti-CDw17) reagents. It always exceeded 95%.

Glycosyl-phosphatidyl Inositol-specific Phospholipase C (PI-PLC) Treatment. PI-PLC purified from *Bacillus thuringiensis* was a kind gift of Dr. M. Low (Columbia University, New York, NY). In a typical preparation, the enzyme had a specific activity of ~1,700 U/ml. Cells (5 × 10^6/ml RPMI–0.25% BSA) were treated with a 1:200 dilution of PI-PLC (45 min, 37°C) and washed twice before testing. Cell viability after treatment was >95% as judged by vital dye exclusion; no loss of specific cell subsets was ever detected as judged by surface marker analysis in indirect immunofluorescence.

Indirect Immunofluorescence. This was performed as previously described in detail (10) using an FITC-goat F(ab')$_2$ anti-mouse Ig (CooperBiomedical) preabsorbed on human IgG. Irrelevant antibodies of matched isotypes were used as negative controls. The samples were analyzed on an Ortho Cytofluorograf 50H connected to a 2150 Data Handling System (Ortho Diagnostic Systems, Inc., Westwood, MA). Intensity of fluorescence was measured on a logarithmic scale.

Immunoprecipitation of FcγRIII(CD16) and N-glycanase Treatment. Intact NK cells, purified by negative selection from either PBL or 10-d cocultures of PBMC with B lymphoblastoid cell lines and PMN from the same donors were labeled with ^{125}I (1 mCi/10^7 cells using 1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglycuroil; (Amersham International, Arlington Heights, IL) (100 μg/tube; Iodogen; Pierce Chemical Co., Rockford, IL). When indicated, ^{125}I-labeled PMN and NK cells were incubated with PI-PLC as described above and both cell-free supernatants and cell pellets were used for immunoprecipitation. After washing, cells were lysed (20 min, 4°C) with 1% NP-40 (Calbiochem-Behring Corp, La Jolla, CA) in 0.1 M Tris, pH 6.8, containing 2 mM EDTA, 2 mM PMSF, 0.33 U/ml aprotinin, 15% glycerol; the cell lysate was centrifuged (13,000 rpm, 30 min) and the postnuclear supernatant was collected. All samples were preabsorbed (12 h, 4°C) with streptavidin-agarose beads (Bethesda Research Laboratories, [BRL] Gaithersburg, MD) (25-μl beads/5 × 10^7 cells) and mouse monoclonal IgG2a of no known antigen specificity (10 μg/5 × 10^7 cells). Aliquots from the samples were sequentially incubated (3 h, 4°C each incubation) with biotin-labeled anti-CD16 or irrelevant antibodies as control (5 μg/10^7 cells) and with Streptavidin-agarose (BRL) (10 μl beads/10^7 cells). The streptavidin-agarose beads were washed 5 times with 0.15 NaCl containing 4 mM EDTA, 1 mM PMSF, 0.02% NaN$_3$, 10 mM Hepes, 0.1% Tween-20, pH 7.2. After boiling (5 min, 100°C) in the presence of 0.5% SDS, 0.1 M 2-ME, each sample was treated with N-glycanase according to the manufacturer. Briefly, sodium-phosphate buffer (0.17 M, pH 8.6), 10 mM 1,10 phenantroline (Sigma Chemical Co., St. Louis, MO), and

1% NP-40 were added to each sample before addition of N-glycanase (Genzyme Corp., Boston, MA) (20 U/ml) to one of two identical aliquots. After an 18-h incubation at 37°C, sample buffer (62.5 mM Tris, pH 6.5, containing 12.5% glycerol, 1% 2-ME, 2.5% SDS, 0.005% bromophenol blue) was added and the samples were analyzed in SDS-10% PAGE (24). Molecular mass markers (Pharmacia Fine Chemicals) were: α-lactalbumin, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, BSA, and phosphorylase b for 14.4, 20.1, 30, 43, 67, and 94 kD, respectively. The gels were dried and exposed to Kodak Xomat x-ray films at −70°C with Lightning Plus intensifying screens (DuPont Co., Wilmington, DE).

RNA and DNA Preparations. NK cells, PMN, and macrophages were washed twice with cold PBS and lysed with 4 M guanidine isothiocyanate for extraction of total cellular RNA after centrifugation through $CsCl_2$ (25). Genomic DNA was prepared from spleen and placenta as previously described (26).

Oligonucleotides. All oligonucleotides were synthesized on an Applied Biosystems Inc. (Foster City, CA) model 381A. HPLC purification was performed according to ABI specifications or oligonucleotides were used without further purification; no differences were observed. The oligonucleotide primers and probes used in this study were derived from the published cDNA sequence for FcγRIII(CD16) (7) and the numbering corresponds to that report.

Oligo 485: GAGAGGCCTGAGGATGAT (870-888); complement
Oligo 491: GGTTGCAAATCCAGAGAA (850-868); complement
Oligo 465: TCATTTGTCTTGAGGGTC (781-799); complement
Oligo 474: TTTCTCCATTTAAGTTTA (761-779); complement
Oligo 488: TTTCTCCATTTAAATTTA (761-779); pos. 766 from NK
Oligo 489: ACAAACATTCGAAGCTCA (724-742); pos. 733 from NK
Oligo 490: ACAAACATTTGAAGCTCA (724-742)
Oligo 473: TGGTACTCCTTTTTGCAG (677-695)
Oligo 466: GTCTCTTTCTGCTTGGTG (658-676)
Oligo 501: AAGAACACTGCTCTGCAT (427-445)
Oligo 494: CACCTGAGGTGTCACAGC (406-424)
Oligo 492: TCTTTGGTGACTTGTCCA (1-18)

cDNA Synthesis and PCR Amplification. 10 μg of total RNA extracted from either PMN or NK cells were incubated in a reaction that contained either 0.5 or 50 pmol of a 3' oligonucleotide primer (485 or 465), 20 U of murine Maloney leukemia virus (MuMLV) reverse transcriptase (Life Sciences, St. Petersburg, FL), 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM $MgCl_2$, and 0.01% gelatin. The reaction was allowed to proceed at 42°C for 60 min, after which time 0.5 or 50 pM of a 5' oligonucleotide primer (492, 494, or 466) was added along with 2.5 units of Taq polymerase (Cetus Corp., Emeryville, CA). 35 cycles of denaturation, annealing, and extension were performed as described (27) in a Perkin Elmer–Cetus Corp. DNA thermal cycler. Denaturation was at 94°C for 1 min, annealing was at 44°C for 2 min, and extension was at 72°C for 3 min. A final cycle with a 7-min extension was performed.

Preparation of ³²P-labeled Fragments and Sequence Analysis. Typically, 20% of a reaction described above was incubated with 5' end-labeled ³²P oligonucleotide internal to the amplified segment of the cDNA, using 50 pM of labeled oligonucleotide. Extension was performed with Taq polymerase (Cetus Corp.) and one cycle of denaturation, annealing, and extension. Denaturation was at 92°C for 1 min, annealing was at 37°C for 2 min, and extension was at 72°C for 10 min. The labeled product was purified on a 5% acrylamide/TBE gel, electro-eluted, and subjected to DNA sequencing using the chemical degradation method (28). Alternatively, the PCR products were cloned into pUC-18 and sequenced by dideoxy chain termination (29).

Oligonucleotide Hybridization of PCR-amplified cDNAs. cDNA from NK cells and PMN obtained from the same donor were synthesized and amplified using oligonucleotides 465/466, spotted on nitrocellulose membranes, denatured, and baked as described (30). 5' end-labeled oligonucleotides specific for NK cells (488, 489) or PMN (474,490) sequences were hybridized and washed as described (31) at 5°C below the calculated T_m.

Isolation of Genomic Clones for FcγRIII(CD16). Eco RI–digested placental DNA was size fractionated on a preparative agarose electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, CA). The 9.0-kb fraction that hybridized with an FcγRIII(CD16) probe was cloned into the phage vector λ CH28, packaged in vitro and plated on *Escherichia coli* strain C600. Nine positive clones were obtained from 150,000 phage that were plaqued purified and analyzed by restriction digestion, oligonucleotide hybridization, and DNA sequence analysis. Similarly, an 18-kb Bam HI fraction, determined to hybridize with the FcγRIII(CD16) probe, was cloned with L47.1. Four positive phage were obtained from 250,000 plaques. Cosmid clones encoding these two genes were isolated from a human placenta cosmid library constructed in the vector pWE15 and generously provided by Dr. Glenn Evans (Salk Institute, San Diego, CA).

Results

Characterization of FcγRIII(CD16) on NK Cells. To define the nature of the FcγRIII(CD16) anchor on NK cells, its presence on buffy coat cells was tested by indirect immunofluorescence after PI-PLC treatment. Fluorescence profiles obtained on the lymphocyte (PBL), in which only NK cells bear FcγRIII(CD16), and PMN populations in the same sample and gated on the basis of their light scatter characteristics are shown in Fig. 1. FcγRIII(CD16) fluorescence, as detected by mAb 3G8, was reduced on both buffy coat and purified PMN by ∼85%, yet little or no decrease for this molecule was observed on PBL. As with fresh lymphocytes (*left panel*), little or no decrease of fluorescence for FcγRIII(CD16) was observed on PI-PLC–treated NK cells purified by negative selection from 10-d cocultures of PBMC with irradiated B lymphoblastoid cell lines (Fig. 1, *right panels*). In contrast, PI-PLC reduced the fluorescence due to LFA-3 in both PMN and NK cell populations by ∼53%. The insensitivity of FcγRIII(CD16) on NK cells, then, is not the result of an inability of these cells to express PI-linked molecules or their insensitivity to PI-PLC because of unique membrane properties of NK cells. These PI-PLC–treated NK cells maintained their characteristic phenotype (NKH-1$^+$/CD5$^-$) and were still able to bind particulate immune complexes (data not shown). These data indicate that FcγRIII(CD16) is resistant to PI-PLC when expressed on NK cells, yet is sensitive to this enzyme when expressed on PMN.

Biochemical Characteristics of FcγRIII(CD16) Precipitated from NK Cells and PMN. The biochemical basis for the altered PI-PLC sensitivity of FcγRIII(CD16) on NK cells was investigated by comparing the protein backbone of the molecule on NK cells and PMN. FcγRIII(CD16) was immunoprecipitated from PI-PLC–treated NK cells and PMN and from the medium in which they were maintained. The products were analyzed on SDS-PAGE after treatment with N-glycanase (Fig. 2). FcγRIII(CD16), which migrates as a broad band of apparent mass 50–70 kD, was immunoprecipitated from control NK cells and PMN (Fig. 2 *A*). N-glycanase treatment of the immunoprecipitate from NK cells resulted in the appearance of two bands of 32 and 36 kD apparent mass, with occasionally a less intense band at 38–40 kD. In contrast, two bands of smaller mass, migrating between 23 and 28 kD, were detected after N-glycanase treatment of the immunoprecipitate from PMN. PI-PLC treatment of NK cells resulted in equivalent amounts of immunoprecipitable FcγRIII(CD16) to those precipitated from untreated control NK cells, while no significant amount of immunoprecipitable protein remained on the PI-PLC-treated PMN (Fig. 2 *B*, cell pellets). In addition to the products described above, N-glycanase treatment gener-
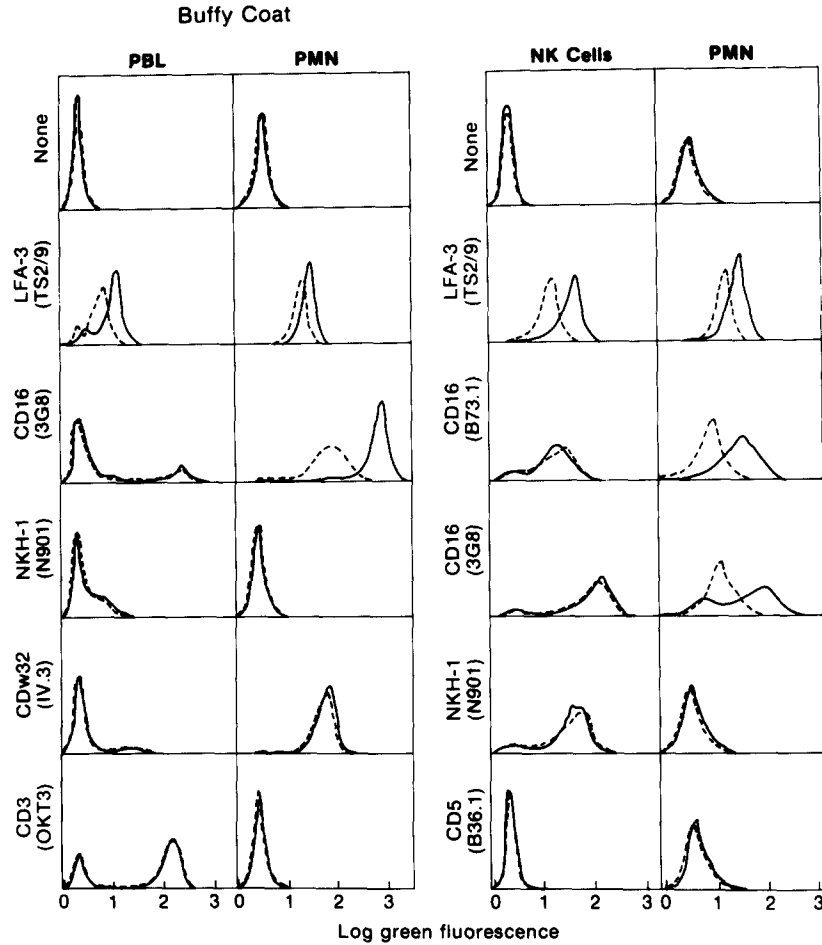
Buffy Coat

FIGURE 1.   FcγRIII(CD16) expression on NK cells and PMN. Buffy coat cells (*left panels*), cultured NK cells, and PMN (*right panels*) were treated with PI-PLC and tested for surface expression of the indicated antigens by indirect immunofluorescence. PBL and PMN in the buffy coat were gated on the basis of their forward and right angle light scatter and fluorescence was measured separately in each region. The histograms in each panel represent intensity of fluorescence: (————) untreated cells; (———) PI-PLC–treated cells. x-axis, intensity of fluorescence (log scale); y-axis, number of cells. The experiment on buffy coat cells is representative of two, those on purified NK cells and PMN are representative of four performed.

ated bands of higher molecular mass that were observed whether N-glycanase digestion was prolonged up to 30 h and using concentrations of the enzyme as high as 40 U/ml (data not shown), as reported previously (11, 14).

Although it is possible that the larger band within the precipitate from each cell type represents an incompletely deglycosylated peptide, in no instance were bands of molecular mass <32 kD or >28 kD precipitated from NK cells or PMN, respectively, in experiments performed with eight different donors. A minimum difference of 4 kD thus exists between the NK cell and PMN FcγRIII(CD16) proteins. Variable but significant amounts of FcγRIII(CD16) were reproducibly immunoprecipi-
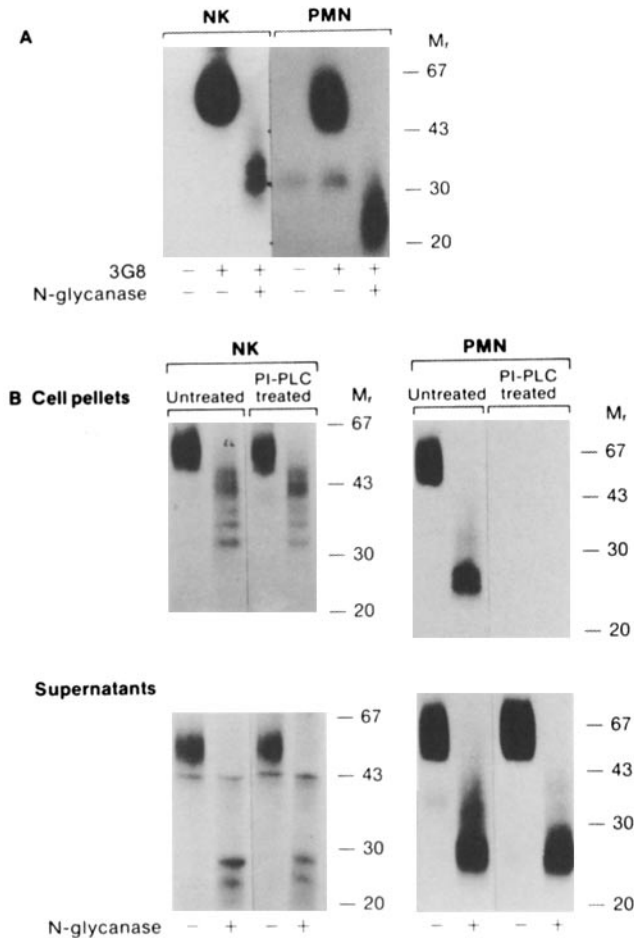
FIGURE 2. PI-PLC-sensitivity and N-glycanase digestion of FcγRIII(CD16). Cultured NK cells purified as described in Materials and Methods and PMN freshly separated from peripheral blood were labeled with [125]I. After labeling, the cells were divided into three aliquots: one of these (control) was processed immediately, one (untreated) was incubated at 37°C in medium, and one (treated) was incubated in the presence of PI-PLC (1:200 dilution, 45 min, 37°C). Antibody 3G8 was used to precipitate FcγRIII-(CD16) from the different cell pellets and from their supernatants, as indicated. Immunoprecipitates, untreated or treated with N-glycanase (20 U/ml, 18 h, 37°C) were analyzed in SDS-10% PAGE. Positions of the molecular weight markers run on the same gels are indicated ($M_r \times 10^{-3}$). (A) 3G8 immunoprecipitates from control cells. In B, lanes labeled cells were exposed three times longer than the corresponding supernatant lanes.

tated from the NK cell supernatant fraction (Fig. 2 B, supernatants) irrespective of PI-PLC treatment. This result was reproducibly obtained using freshly isolated NK cells or B73.1 antibody for FcγRIII(CD16) precipitation (data not shown). In agreement with a previous report (16), significant amounts of protein were also detected in the supernatant fraction from untreated PMN, which was more abundant in the supernatants from PI-PLC–treated PMN. N-glycanase treatment of the immunoprecipitates from supernatants of both NK cells and PMN resulted in two bands of 23 and 28 kD apparent molecular mass irrespective of PI-PLC treatment. These results are in agreement with a previous study (7) showing that the FcγRIII(CD16) polypeptides precipitated from PI-PLC–treated NK cells supernatants, representing ~50% of those present on control cells, had apparent molecular masses of 28 kD after N-glycanase treatment. Precipitation from NK cell pellets or control supernatants was not reported in that study. It is most likely that the soluble form of FcγRIII(CD16) precipitated from the NK cell supernatants is not the result of PI-hydrolysis and, instead, derives from proteolytic cleavage of the molecule at a posi-

tion, near the transmembrane domain, at which the molecule is processed in PMN during formation of the PI anchor. A similar finding has been reported for the low affinity FcεRII. An IgE-binding, 37-kD molecule, detected in the supernatant of FcεRII-expressing human B cell lines (32) arises by proteolytic degradation of a 45-kD transmembrane form of FcεRII. These observations on the differences in the PI-PLC sensitivity and molecular weight after deglycosylation of FcγRIII(CD16) on NK cells and PMN prompted us to characterize the FcγRIII(CD16) gene and its transcript in NK cells and PMN.

Analysis of FcγRIII(CD16) RNA in NK Cells and PMN. The molecular basis for the structural differences observed for FcγRIII(CD16) on PMN and NK cells was approached through the analysis of the RNA encoding these molecules. Northern blot analysis using FcγRIII(CD16)-specific probes revealed a single species of identical size in PMN and NK cells (not shown). In a previous study (7), S_1 analysis demonstrated no detectable differences in the FcγRIII(CD16) encoding RNA obtained from NK cells as compared with a cDNA sequence encoding the PI-linked molecule. To determine the structural basis for the size difference of the deglycosylated proteins observed on PMN and NK cells, sequence analysis of cDNAs corresponding to these RNA was performed. Total RNA was extracted from both cell types obtained from an NA-2/NA-2 donor, converted to cDNA using FcγRIII(CD16)-specific primers and reverse transcriptase and amplified by the polymerase chain reaction. Sequence analysis of those cDNA revealed single nucleotide substitutions in the NK cell transcript (corresponding to nucleotides 1-887). For example, as shown in Fig. 3 *A*, a T at position 733 in the sequence derived from PMN RNA is seen to be a C in the sequence obtained from NK cell RNA, resulting in an extended open reading frame for the NK cell transcript. Similarly, a C at position 766 in PMN is found to be a T in NK cells. Sequence analysis of this region of the FcγRIII(CD16) RNA obtained from the cDNA amplified from these cell types of this donor revealed the following cell type-specific single nucleotide substitutions: position 141 (C to G), 147 (T to C), 277 (A to G), 473 (A to G), 505 (C to T), 531 (T to C), 559 (G to T), 641 (C to T), 733 (T to C), 766 (C to T), 814 (A to G), and 829 (G to A) (Fig. 3 *B*). These 12 nucleotide changes result in six amino acid changes (Fig. 3 *B*). Similar sequence analysis of transcripts derived from PMN and NK cells of a second donor (NA-1/NA-1) revealed the same nucleotide substitutions between NK cells and PMN at positions 473, 505, 531, 559, 641, 733, 766, 814, and 829 as were seen for the NA-2/NA-2 donor, revealing that these differences between NK cells and PMN were not the result of allelic variation. In contrast, five nucleotides were found to differ between PMN transcripts of NA-2 and NA-1 donors: positions 141 (C→G), 147 (T→C), 227 (G→A), 277 (A→G), and 349 (A→G) (bold-faced nucleotides in III-1, Fig. 3 *B*). No differences were detected in NK transcripts between NA-2 and NA-1, indicating that NA-1 and NA-2 alleles are restricted to III-1. The three nucleotide differences at positions 141, 147, and 277 coincide with the cell type-specific differences between III-1 and III-2 in the NA-2 homozygous donor, resulting in a III-1, NA-1 sequence identical to III-2 at those positions. This pattern of nucleotide substitution in III-1 for NA-1 and NA-2 allows for the mapping of these epitopes. Since NK cells are always NA-2$^+$ and do not express the NA-1 epitope, we can conclude that the nucleotide differences at positions 227 and 349 must determine the NA-1 and NA-2 epitopes. The G→A transition in III-1, NA-1 at position 227 results

FIGURE 3. Sequence analysis of FcγRIII(CD16) transcripts in PMN and NK cells. (*A*) RNA extracted from the indicated cells of a single individual were converted to cDNA using oligo 465 and amplified by PCR using oligo 466 (see Materials and Methods). Oligo 473 was end labeled with ^{32}P and used to generate an extension product from the amplified cDNA, gel purified, and sequenced by the chemical degradation method (28). Identical results were obtained using oligos 485/494 on total RNA, extending with oligo 466 or 473. Sequences were confirmed on the opposite strand using oligos 491, 465, or 474. Asterisks indicate nucleotide differences between the cell types. The effect of these sequence changes on the translation of the FcγRIII(CD16) transcript is indicated. The lanes of the sequencing gels are (left to right): G, A>C, T+C, and C. (*B*) Nucleotide sequence of cDNA for FcγRIII(CD16) obtained from NK cells and PMN of an NA-2/NA-2 individual. III-1 indicates the PMN sequence; III-2 the NK cell sequence. Identical nucleotides are indicated by dashes. III-1 nucleotides indicated in bold-faced type are allelic in NA-1/NA-1; overlined nucleotides (227 and 349) determine the NA-2 and NA-1 reactivities of PMN FcγRIII(CD16), respectively. The predicted amino acid sequence is shown below with the hydrophobic core of the signal sequence and transmembrane domain overlined. N-linked glycosylation sites are underlined. The extended reading frame for the NK cell transcript (III-2) is indicated in bold-faced type, as are the amino acid differences.

B

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III-1 TCTTTGGTGACTTGTCCACTCCAGTGTGGCATCATGTGGCAGCTGCTCCTCCCAACTGCTCTGCTACTTCTAGTT 75
III-2 ---------------------------------------------------------------------------

III-1 TCAGCTGGCATGCGGACTGAAGATCTCCCAAAGGCTGTGGTGTTCCTGGAGCCTCAATGGTACAGCGTGCTTGAG 150
III-2 ----------------------------------------------------------G----C---

III-1 AAGGACAGTGTGACTCTGAAGTGCCAGGGAGCCTACTCCCCTGAGGACAATTCCACACAGTGGTTTCACAATGAG 225
III-2 ---------------------------------------------------------------------------

III-1 AGCCTCATCTCAAGCCAGGCCTCGAGCTACTTCATTGACGCTGCCACAGTCAACGACAGTGGAGAGTACAGGTGC 300
III-2 ----------------------------------------------------G---

III-1 CAGACAAACCTCTCCACCCTCAGTGACCCGGTGCAGCTAGAAGTCCATATCGGCTGGCTGTTGCTCCAGGCCCCT 375
III-2 ---------------------------------------------------------------------------

III-1 CGGTGGGTGTTCAAGGAGGAAGACCCTATTCACCTGAGGTGTCACAGCTGGAAGAACACTGCTCTGCATAAGGTC 450
III-2 ---------------------------------------------------------------------------

III-1 ACATATTTACAGAATGGCAAAGACAGGAAGTATTTTCATCATAATTCTGACTTCCACATTCCAAAAGCCACACTC 525
III-2 -----------------------G-----------------------T---

III-1 AAAGATAGCGGCTCCTACTTCTGCAGGGGGCTTGTTGGGAGTAAAAATGTGTCTTCAGAGACTGTGAACATCACC 600
III-2 ------C---------------------------T---

III-1 ATCACTCAAGGTTTGGCAGTGTCAACCATCTCATCATTCTCTCCACCTGGGTACCAAGTCTCTTTCTGCTTGGTG 675
III-2 ----------------------------------T---

III-1 ATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTCTGTGAAGACAAACATTTGAAGCTCAACAAGAGAC 750
III-2 -----------------------------------------------C---

III-1 TGGAAGGACCATAAACTTAAATGGAGAAAGGACCCTCAAGACAAATGACCCCCATCCCATGGGAGTAATAAGAGC 825
III-2 --------------------T---------------------------------G---

III-1 AGTGGCAGCAGCATCTCTGAACATTTCTCTGGATTTGCAACCCCATCATCCTCAGGCCTCTC 887
III-2 ---A---
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III-1 MWQLLLPTALLLLVSAGMRTEDLPKAVVFLEPQWYSVLEKDSVTLKCQGAYSPEDNSTQWFHNESLISSQASSYF 75
III-2 ------------------------------------------------R---

III-1 IDAATVNDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPRWVFKEEDPIHLRCHSWKNTALHKVTYLQNGKDRKY 150
III-2 ------D-----------------------------------------------------G---

III-1 FHHNSDFHIPKATLKDSGSYFCRGLVGSKNVSSETVNITITQGLAVSTISSFSPPGYQVSFCLVMVLLFAVDTGL 225
III-2 -------Y-------------------F---------------------F---

III-1 YFSVKTNI*                       233
III-2 -------RSSTRDWKDHKFKWRKDPQDK*    254
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in the loss of the glycosylation sequence Asn-Glu-Ser[65] in III-1, NA-2, converting it to Asn-Glu-Asn[65]. Similarly, the A→G transition at position 349 in III-1, NA-1 results in an Ile[106] to Val[106] change. III-2, although not allelic for NA-1/NA-2, has been found to be allelic at nucleotide 559 (T/G), as determined from the sequencing of the III-2 gene and transcript from 6 donors (not shown).

The specificity of expression of the III-1 and III-2 transcripts was demonstrated by oligonucleotide hybridization. Oligonucleotide probes that differed only at a single nucleotide, indicated in Fig. 4, were hybridized to PCR-amplified cDNA obtained from four sets of donor-matched PMN and NK cells. In all cases, the NK cell–derived RNA contain a C at position 733 and a T at position 766, while PMN have a T and C at these positions, respectively. An oligonucleotide probe common to both cell type transcripts hybridized to all samples. Hybridization and sequencing studies revealed no evidence for molecular heterogeneity within a single cell type.

Structural Analysis of Two FcγRIII(CD16) Genes.   The basis for these single nucleotide differences in FcγRIII(CD16) transcripts of NK cells and PMN was investigated by determining the gene structure for this receptor. Southern blot analysis of placenta or spleen DNA restricted with Eco RI, Hind III, Nco I, Kpn I, Bgl II, and Pst I and probed with exon-specific probes for the signal sequence, the extracellular do-
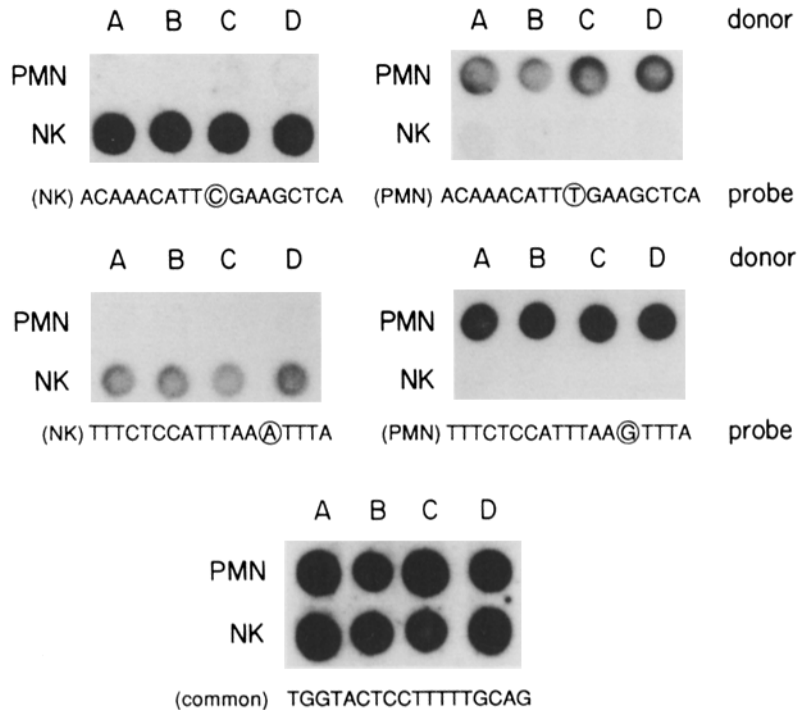

FIGURE 4.   Oligonucleotide hybridization of PCR-amplified cDNA of NK cells and PMN. RNA extracted from NK cells or PMN of single individuals was converted to cDNA and amplified using oligos 465/466, spotted on nitrocellulose and hybridized with end-labeled, NK-specific oligos 488 and 489 or PMN-specific oligos 474 and 490 or the FcγRIII(CD16) common oligo 473. Four donors were studied, indicated A–D. The single nucleotide difference in each oligo is indicated.

mains (encoded on two exons), or the transmembrane-cytoplasmic-3' UT exon all demonstrated single restriction fragments (not shown), suggestive of a single gene or two highly conserved genes. However, Bam HI revealed two restriction fragments of 4.8 and 18 kb when probed with either an EC-2 probe or TM-CYT-3' UT probe (Fig. 5). Similarly, Hinc II revealed two fragments of 5.0 and 3.0 kb when probed with this probe (not shown). To rule out polymorphism as the basis for these additional fragments, DNA from 23 individuals of different racial origins were restricted with Hinc II and probed with the FcγRIII(CD16) TM-CYT-3' UT probe. All DNAs revealed two distinct Hinc II fragments (not shown). These two distinct, but highly homologous genes for FcγRIII(CD16) were cloned by size fractionation of Eco RI–restricted placental DNA and cloning of the 9.0-kb fragment that encodes each FcγRIII(CD16) gene. Nine independent FcγRIII(CD16) clones were obtained which were screened with oligonucleotide probes described in Fig. 4 specific for each transcript. Five clones hybridized only with the NK-specific oligonucleotides, while four clones hybridized exclusively with the PMN-specific oligonucleotides. In addition to these clones, the 18-kb Bam HI fragment shown in Fig. 5 was cloned and, as expected, hybridized only with the NK-specific oligonucleotide probes. Cosmid clones for each of these genes were also obtained. DNA sequence analysis of these two classes of clones confirmed the hybridization results. Gene III-1 encodes the PMN transcript sequence, while gene III-2 encodes the NK cell transcript sequence. The III-1 sequence determined demonstrates the five nucleotide substitutions in EC-1 characteristic of the NA-1 allele. Further evidence for a gene duplication for FcγRIII(CD16) was obtained by utilizing the observation that the nucleotide changes at position 733 and 766 each generate a novel restriction enzyme recognition site. The T→C change at 733 creates a Taq I site, while the C→T change at 766 creates a Dra I site. As seen in Fig. 5, Taq I or Dra I digestion of spleen DNA hybridized with TM or 3'UT probes reveals the existence of both sequences in the genome. When probed with the TM probe, Taq I reveals fragments of 6.5, 3.8, 3.5 kb (3.8- and 3.5-kb bands migrate as a doublet in this experiment) and 280 bp (seen optimally with the 3' UT probe), while Dra I generates fragments at 900, 550, and 350 bp. The 6.5-kb Taq I fragment seen in this experiment represents a polymorphism as determined by screening DNA obtained from individuals of different racial origins with Taq I. It is associated with the III-1 NA-2 allele, as determined by Taq I digestion of DNA derived from NA-1/NA-1, NA-2/NA-2, and NA-1/NA-2 donors and hybridizing with the FEγRIII TM/CYT probe (not shown). The 550-bp Dra I fragment is not detected when the 3' UT probe is used, since the probe is 3' of the expected fragment. The additional Taq and Dra fragments of 3.0 and 3.2 kb, respectively, detected with the 3' UT probe result from corresponding 3' fragments. Cloned genes specific for each transcript, when probed with the TM sequence, produce either a 3.8-kb Taq fragment (III-1, NA-1) or a 3.5- and 0.28-kb fragment (III-2); Dra I reveals either an 900-bp fragment (III-1) or a 550- and 350-bp fragment (III-2). A map of these two genes, derived from hybridization and sequence analysis, is presented in Fig. 5.

The Nucleotide Substitution at Position 733 Eliminates a Translation Termination Sequence. The nucleotide change at position 733 in gene III-2 occurs at a position in gene III-1 that specifies an in frame translation termination codon TGA (occurring after the codon for amino acid 233), encoding in its place a CGA codon that specifies the amino acid arginine. The resulting open reading frame for the transcript de-
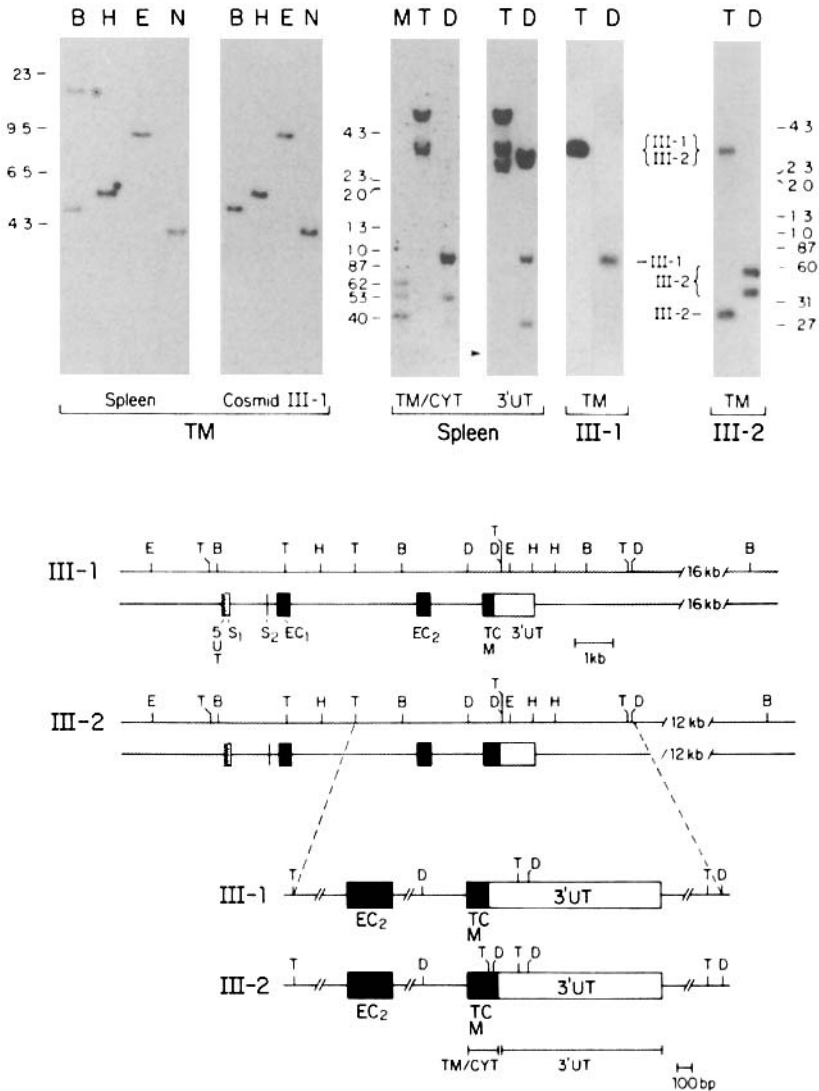
FIGURE 5.   Two genes encode FcγRIII(CD16): III-1 and III-2. (*Top*) Southern blot analysis of spleen DNA or DNA derived from clones of each gene as indicated. The blots were probed with ^{32}P-labeled probes as indicated under each autoradiograph. The 18-kb Bam HI fragment detected with the TM probe, encoded on the III-2 gene, is denoted with an asterisk. An arrow indicates the 280-bp Taq I fragment derived from the III-2 gene. The Taq and Dra experiments were resolved on 1.2% agarose gels to optimize the separation of small fragments, the blots marked spleen were probed first with the TM probe, stripped, and reprobed with the 3' UT probe. (*Bottom*) Restriction maps of the two FcγRIII(CD16) genes. The exon-intron structure of each gene is indicated, as determined by DNA sequence analysis. The region corresponding to the TM-CYT-3'UT exon is expanded to indicate the position of the Taq I and Dra I sites generated in III-2 by the nucleotide differences between these genes (these sites are not indicated for III-2 on the upper map for clarity). Probes used in this study are indicated below the maps. E, EcoRI; B, Bam HI; H, Hind III; T, Taq I; D, Dra I; N, Nco I; M, labeled markers. The restriction maps are not complete for the sequences flanking these genes 5' of the signal exon and 3' of the 3' UT exon.

A


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              229 233
192    209    )  (         254
     ┌──────┬─────┬─┬──────────────────┐
     │      │ TM  │C│    3'UT          ≷
     └──────┴─────┴─┴──────────────────┘
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                G  L  A  V  S  T  I  S  S  F  S  P  P  G  Y  Q  V  S  F  C  L  V
III-1    .........GTTTGGCAGTGTCAACCATCTCATCATTCTCTCCACCTGGGTACCAAGTCTCTTTCTGCTTGGTG
PMN      .........GUUUGGCAGUGUCAACCAUCUCAUCAUUCUCUCCACCUGGGUACCAAGUCUCUUUCUGCUUGGUG
III-2    .........GTTTGGCAGTGTCAACCATCTCATCATTCTCTCCACCTGGGTACCAAGTCTCTTTCTGCTTGGTG
NK       .........GUUUGGCAGUGUCAACCAUCUCAUCAUUCUUUCCACCUGGGUACCAAGUCUCUUUCUGCUUGGUG
                                               F
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          M  V  L  L  F  A  V  D  T  G  L  Y  F  S  V  K  T  N  I  *
III-1    ATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTCTGTGAAGACAAACATTTGAAGCTCAACAAGAGAC
PMN      AUGGUACUCCUUUUUGCAGUGGACACAGGACUAUAUUUCUCUGUGAAGACAAACAUUUGAAGCUCAACAAGAGAC
III-2    ATGGTACTCCTTTTTGCAGTGGACACAGGACTATATTTCTCTGTGAAGACAAACATTCAAAGCTCAACAAGAGAC
NK       AUGGUACUCCUUUUUGCAGUGGACACAGGACUAUAUUUCUCUGUGAAGACAAACAUUCGAAGCUCAACAAGAGAC
                                                        R  S  S  T  R  D
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III-1    TGGAAGGACCATAAACTTAAATGGAGAAAGGACCCTCAAGACAAATGACCCCCATCCCATGGGAGTAATAAGAGC
PMN      UGGAAGGACCAUAAACUUAAAUGGAGAAAGGACCCUCAAGACAAAUGACCCCCAUCCCAUGGGAGUAAUAAGAGC
III-2    TGGAAGGACCATAAATTTAAATGGAGAAAGGACCCTCAAGACAAATGACCCCCATCCCATGGGGTAATAAGAGC
NK       UGGAAGGACCAUAAAUUUAAAUGGAGAAAGGACCCUCAAGACAAAUGACCCCCAUCCCAUGGGGGUAAUAAGAGC
          W  K  D  H  K  F  K  W  R  K  D  P  Q  D  K  *
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III-1    AGTGGCAGCAGCATCTCTGAACA.....
PMN      AGUGGCAGCAGCAUCUCUGAACA.....
III-2    AGTAGCAGCAGCATCTCTGAACA.....
NK       AGUAGCAGCAGCAUCUCUGAACA.....
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B

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                    216                                    261
Mouse FcγRIIα       AFSLVHCLLFAVDTGLYFYVRRNLQTPREYWRKSLSIRKHQAPQDK*
                    .:.::: ::::::::::: :. :..... : :.. ... ..::::
Human FcγRIII-NK    SFCLVHVLLFAVDTGLYFSVKTNIRSSTRDW-KDHKFKWRKDPQDK*
                    209                                    254
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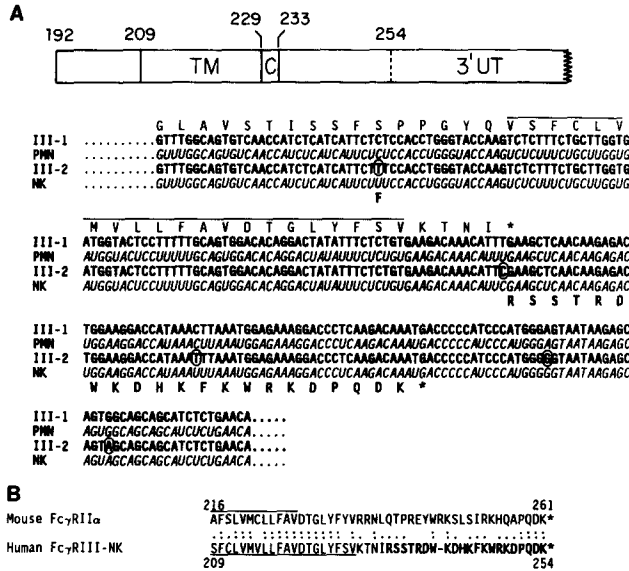
FIGURE 6. Sequence comparison of FcγRIII(CD16) transcripts in NK cells, PMN and genomic DNA. (A) Nucleotide sequence data are shown for the III-1 and III-2 genes and their deduced transcripts for the transmembrane/cytoplasmic/3' UT exon (indicated schematically at the top of the figure with relevant amino acids numbered) beginning at nucleotide 611 (numbering according to the published cDNA sequence [7]), which is the first nucleotide of this exon and extending to nucleotide 848. The genomic sequences were obtained independently from the cloned FcγRIII(CD16) genes isolated as described (see text and Materials and Methods). Positions where the sequences were found to differ are circled in the III-2 sequence. The predicted translation is indicated, with the extended reading frame for III-2 gene and NK cell transcript indicated in bold-faced type. (B) Sequence alignment of the predicted COOH terminus of Fcγ-RIII(CD16) in NK cells compared with the murine homologue, FcγRIIα, using the fastp algorithm (37). Two dots indicate identity, single dots indicate changes that arise by single nucleotide substitutions. The transmembrane domain is indicated.

rived from III-2, shown in Fig. 6 *a*, encodes an additional 21 amino acids, terminating at nucleotide 797. The predicted cytoplasmic domain for the FcγRIII(CD16) sequence transcribed from the III-2 gene in NK cells is 25 amino acids long. The 46 additional amino acids encoded by the transmembrane and cytoplasmic domains present in the III-2 transcript in NK cells could account for an additional 6,000 daltons of mass, as would be predicted for a transmembrane-anchored FcγRIII(CD16) protein of NK cells. This value is in agreement with the results presented in Fig. 2, indicating that FcγRIII(CD16) on NK cells is resistant to PI-PLC and migrates, when deglycosylated, as a protein of apparent mass 5–10,000 dalton larger than its PMN homologue. The homology between the FcγRIII(CD16) protein predicted for human NK cells and the murine FcγRIIα molecule is increased by this extended open reading frame. The FcγRIII(CD16) sequence for the NK cell molecule now demonstrates homology to the mouse FcγRIIα molecule not only in its transmembrane domain, as has been observed (7), but in its cytoplasmic domain as well (Fig. 6 *b*).

Discussion

FcγRIII(CD16) of NK cells and PMN differ in their PI-PLC sensitivity and their apparent molecular weights after deglycosylation. Cell type–specific transcripts that differ by single nucleotide substitutions were found to encode these alternative forms of FcγRIII(CD16). Numerous examples of alternative splicing to generate alternatively anchored or secreted proteins have been described (N-CAM, DAF, AchE and

LFA-3; reviewed in reference 17). The molecular basis for the structural differences between the FcγRIII(CD16) molecule on PMN and NK cells is the result of cell type–specific transcription of nearly identical but distinct genes that encode these single nucleotide differences.

cDNA for FcγRIII(CD16) expressed in NK cells and PMN were synthesized and then amplified by the polymerase chain reaction. These sequences were found to differ by multiple single nucleotide substitutions found in regions of the molecule corresponding to the extracellular domain and transmembrane-cytoplasmic-3' UT regions. That these differences were observed consistently in only one (NK) of the paired RNA (NK/PMN) samples processed simultaneously argues against reverse transcriptase or PCR amplification as the source of these differences. Polymorphism has been reported to be associated with FcγRIII(CD16) on PMN. Two allelic forms (NA-1 and NA-2) have been described that are distinguished by human alloantisera, by molecular mass in SDS-PAGE, and by specific mAbs (21). Five nucleotide changes in the EC-1 domain of the III-1 gene are associated with allelic forms of the molecule, two of which are specific for the NA-1 or NA-2 alleles (227 and 349). The FcγRIII(CD16) protein immunoprecipitated from NA-2 homozygous donors migrates more slowly on SDS-PAGE as compared to NA-1 allele. The $Ser^{65} \rightarrow Asn^{65}$ change that results in the loss of a glycosylation site in NA-1 is therefore consistent with Ser^{65} determining the NA-2 allele. The Val^{106}, then, is a likely candidate for the NA-1 determinant. The remainder of the changes were observed in different cell types of the same individual and were consistently observed in all NK cell samples, regardless of donor allele. Two genes encoding FcγRIII(CD16) were cloned and characterized. Both genes are encoded on human chromosome 1, tightly linked to FcγRII(CD32) genes and each other (Qiu, W. Q., R. Pearse, and J. Ravetch, unpublished observations). These genes have nearly identical restriction maps and encode all the sequence differences observed between the NK cell and PMN transcripts for the appropriate allele. A 6.5-kb Taq fragment was found to be polymorphic and specifically associated with the III-1, NA-2 allele; this band was not detected in a cosmid clone encoding the III-1 gene of the NA-1 allele (Fig. 5). This polymorphism should prove useful in the rapid typing of NA-1 and NA-2 alleles of III-1. The nonallelic nucleotide differences between III-1 and III-2 occur in all exons encoding the receptor, ruling out alternative splicing as the mechanism generating the alternative anchored forms of this receptor. However, the degree of identity between these genes is remarkable. This high degree of identity suggests either a recent evolutionary history or a mechanism-like gene conversion that maintains sequence fidelity.

Cell type–specific transcription of these two genes, III-1 in PMN and III-2 in NK cells, appears to be exclusive (Fig. 4) and results in the appearance of alternatively anchored forms of the FcγRIII(CD16) protein. The basis for this alternative anchoring results from the differences between these two genes and their respective transcripts. The III-1 gene encodes a transcript with a short (four amino acid) cytoplasmic domain, which is expressed as a PI-anchored protein both in PMN and transfected COS cells. III-2 gene encodes a transcript with a cytoplasmic domain of 25 amino acids as a result of a T→C substitution in the termination codon TGA of the III-1 gene. The effect of this longer cytoplasmic domain is likely to contribute to the processing of this protein from a PI-linked molecule to a transmembrane pro-

tein. This hypothesis is supported by our studies on murine FcγRIIα, which is homologous to the FcγRIII(CD16) predicted for NK cells in its transmembrane and cytoplasmic domains. Murine FcγRIIα can be transfected and expressed in murine L cells (33), which do not express PI-linked molecules (34). In addition, we have observed that FcγRIIα is expressed as a PI-PLC resistant molecule on mouse macrophage cell lines (Zalman, H., and J. Ravetch, unpublished) and NK cells (35).

Possible Functions for the Alternatively Anchored Forms of FcγRIII(CD16). FcγRIII(CD16) has been postulated to mediate different functions on NK cells and PMN. This FcγR on NK cells mediates ADCC; the interaction of FcγRIII(CD16) with ligand on NK cells results in transduction of intracellular signals, presumably through its intracytoplasmic domain, that induce activation of genes for lymphokines and receptors involved in NK cell functions and biology (23, 36). This activation is mediated, at least partly, through increased [Ca^{2+}]$_i$ and receptor-induced PI hydrolysis. The role of FcγRIII(CD16) on PMN is less certain. Those cells express two distinct classes of receptors for immune complexes, FcγRII(CD32) and FcγRIII(CD16). Antibodies to either FcγR class inhibit PMN functions, suggesting that both receptors may be needed to trigger a functional response and may act synergistically when presented with multivalent immune complexes. Since PMN anchor FcγRIII(CD16) with a PI tail attachment, it has been proposed that the role of this form of the molecule is to capture immune complexes without triggering neutrophil activation (13). The cell type–specific expression of the two genes encoding FcγRIII(CD16) described here that generates these alternative protein forms thus has a significant effect on the biological function of these molecules.

Summary

A low affinity receptor for IgG immune complexes, FcγRIII(CD16), is expressed on human NK cells as an integral membrane glycoprotein anchored through a transmembrane peptide; on polymorphonuclear neutrophils (PMN) the receptor is anchored through a phosphatidylinositol (PI) linkage. The protein on NK cells has a molecular mass 6–10 kD larger than that on PMN, and, unlike the latter, is resistant to PI-specific phospholipase C (PI-PLC). FcγRIII(CD16) transcripts isolated from PMN and NK cells of single donors revealed multiple single nucleotide differences, one of which converts an in frame UGA termination codon to a CGA codon. The resulting open reading frame encodes a longer cytoplasmic domain for FcγRIII(CD16) in NK cells, contributing to its transmembrane anchor. Two nearly identical, linked genes that encode these transcripts have been cloned for FcγRIII(CD16), one of which (III-1) is allelic for NA-1 and NA-2. The allelic sites have been mapped to two single nucleotides in the extracellular domain. These genes are transcribed in a cell type–specific fashion to generate the alternatively anchored forms of this receptor.

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