cytoplasmic domain in LFA-3 (*16–19*), or completely different cytoplasmic, transmembrane, and extracellular amino acid sequences in the neural cell adhesion molecule (*24, 25*). Serine and other small hydrophilic side chain amino acid residues are commonly found at and near the site of PIG anchor attachment (*20*). However, their role in regulating anchor attachment had not been appreciated, although it is known that a segment including the 20 amino acids in the extracellular domain, together with the transmembrane domain, are required to signal reanchoring of decay accelerating factor (*26*). The studies here further illuminate the relative dominance of protein sequence and post-translational mechanisms, including subunit association and availability of the appropriate enzymes for PIG assembly and attachment in regulating PIG reanchoring.

We have not demonstrated association in NK cells of CD16 and the γ subunit, although the requirement for coexpression in COS or L cells and the presence of a γ subunit of identical sequence in NK cells is suggestive. A mouse IgG Fc receptor, FcγRIIa, also requires γ chain for cell surface expression (*27*) as does the FcεRI (*28*). For these receptors, demonstration of γ association requires special conditions. This may explain previous (*5–7, 29*), and our own (unpublished), lack of identification of this subunit in association with CD16 in NK cells. The γ subunit has little exposure on the outside of the cell (*12*). Lack of significant homology in cytoplasmic domains of CD16, FcεRI, and mouse FcγRIIa, together with lack of requirement of the CD16 cytoplasmic domain for association, suggests association is governed primarily by the transmembrane region. The γ subunit is related to the T cell receptor ζ subunit (*30*) and may participate in related signaling pathways. Recently, ζ has also been shown to be expressed in NK cells (*31*) and is also a candidate for a subunit that may associate with CD16.

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Membrane Anchoring of a Human IgG Fc Receptor (CD16) Determined by a Single Amino Acid

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CD16 is a low-affinity immunoglobulin G (IgG) Fc receptor that is expressed on natural killer (NK) cells, granulocytes, activated macrophages, and some T lymphocytes. Two similar genes, CD16-I and CD16-II, encode membrane glycoproteins that are anchored by phosphatidylinositol (PI)-glycan and transmembrane polypeptides, respectively. The primary structural requirements for PI-linkage were examined by constructing a series of hybrid cDNA molecules. Although both cDNA's have an identical COOH-terminal hydrophobic segment, CD16-I has Ser^{203} whereas CD16-II has Phe^{203}. Conversion of Phe to Ser in CD16-II permits expression of a PI-glycan–anchored glycoprotein, whereas conversion of Ser to Phe in CD16-I prevents PI-glycan linkage.

THE CD16 IGG FC RECEPTOR (FcγRIII) is identified by monoclonal antibodies (MAb) 3G8, B73.1, Leu 11, and others (*1*). The binding of immune complexes or anti-CD16 MAb to NK cells causes a rapid increase in intracellular Ca^{2+} and inositol triphosphate generation, with subsequent transcription of lymphokines and triggering of cell-mediated cytotoxicity (*2, 3*). In contrast, on granulocytes CD16 may function simply as a binding structure for IgG complexes (*3, 4*).

Biochemical differences between CD16 on NK cells and granulocytes may account for these functional distinctions (*3*). The CD16 polypeptide on granulocytes is ~30 kD, but is ~36 and ~38 kD on NK cells. CD16 on granulocytes is susceptible to cleavage with phosphatidylinositol phospholipase C (PI-PLC), indicating phosphatidylinositol (PI)-glycan linkage, whereas CD16 on NK cells is resistant to PI-PLC (*5*). CD16 is diminished on granulocytes but is unaffected on macrophages in patients with paroxysmal nocturnal hemoglobinuria, a disease selectively affecting PI-anchored proteins (*6*). Two genes exist for CD16 (*7*). Granulocytes and NK cells preferentially transcribe CD16-I and CD16-II, respectively (*7, 8*). We examined the structural requirements for PI linkage of CD16-I.

A λ gt11 cDNA library from human NK

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cells cultured in recombinant interleukin-2 was screened with pCD16-I cDNA (*8*). A second form of cDNA (CD16-II) was identified. The presence of an Arg residue (CGA) at codon 234 in the NK cell–derived cDNA, instead of a termination codon (TGA), allows for an additional 21 amino acids in the cytoplasmic domain of the polypeptide, prior to the termination site at codon 255 (Fig. 1). The presence of Arg^{36}, Asp^{82}, Gly^{147}, Tyr^{158}, and Phe^{203} are in accordance with the CD16-II sequence recently reported (*7*). There were two other substitutions noted at codons 66 (His) and 245 (Phe) and two silent changes at codons 38 (CTT to CTC in CD16-II) and 166 (GAT to GAC in CD16-II). The difference at codon 82 should result in the presence of a Sal I site in CD16-II (GTCGAC), but not CD16-I (GTC*A*AC) (*8*). However, pCD16-I cDNA is cleaved by Sal I, suggesting a possible mistake in the original sequence or polymorphism at this site (*7*). This is significant, since an Asn^{82} in CD16-I would provide a potential site for N-linked glycosylation.

Since the most obvious difference between CD16-I and CD16-II is at residue 234, this codon was converted from TGA (stop) to AGG (Arg) by site-directed mutagenesis of pCD16-I (Fig. 1). COS-7 cells were transfected with pCD16-I.AGG plasmid, treated (or not) with PI-PLC, stained with anti-CD16 MAb and analyzed by flow cytometry (Fig. 2A). PI-PLC removed >90% of the membrane CD16. Similarly, a hybrid CD16 construction (pCD16-I.Kpn I–3′ II) that contained CD16-I 5′ of the Kpn I site and CD16-II 3′ of the Kpn I site (Fig. 1), also produced a PI-glycan–linked CD16 (Fig. 2A). This was not cell type–restricted. Transfectants were established in the human T leukemia cell Jurkat with CD16-I.AGG and CD16-I.Kpn I–3′ II. PI-PLC treatment removed essentially all CD16 from the cell surface (Fig. 2B). The Arg^{234} residue in pCD16-I.Kpn I–3′ II was CGA, whereas the Arg^{234} in pCD16-I.AGG was AGG. This excluded the possibility that the COS-7 or Jurkat cells converted the CD16-II CGA to a termination codon by a single base conversion event at a post-transcriptional stage, as observed with apolipoprotein B (*9*).

A likely candidate for affecting membrane anchoring was the difference at residue 203 (CD16-I, Ser; CD16-II, Phe). Since the Pst I–Kpn I fragments of the CD16-I and CD16-II cDNA differ only at this residue, this provided a convenient method for Phe^{203}–Ser^{203} conversion (Fig. 1). The Pst I–Kpn I fragment of pCD16-II was subcloned into pCD16-I.AGG, and, reciprocally, the Pst I–Kpn I fragment of pCD16-I was inserted into pCD16-II which created pCD16-I.AGG.Phe^{203} and pCD16-II.Ser^{203}, respectively. pCD16-II, pCD16-I.AGG.Phe^{203}, and pCD16-II.Ser^{203} were transfected into COS-7 cells and analyzed for expression and membrane anchoring of CD16. COS-7 cells transfected with pCD16-II did not express CD16 on the cell surface, despite the presence of abundant transcripts and protein in the cytoplasm. However, conversion of Phe^{203} to Ser^{203} enabled membrane expression (Fig. 3). CD16 was readily cleaved from the surface of the pCD16-II.Ser^{203} transfectant by PI-PLC. By contrast, CD16 was not expressed on the surface of COS-7 cells transfected

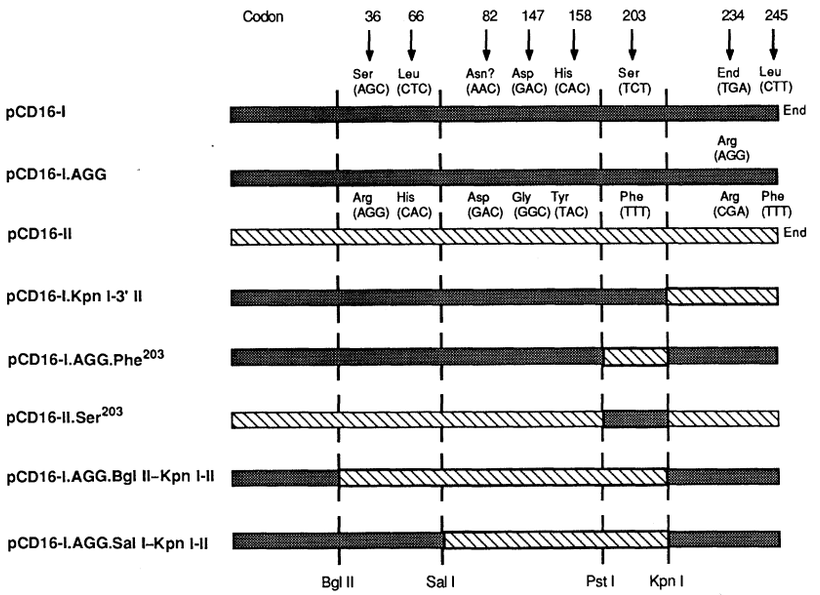

Fig. 1. CD16-I and CD16-II cDNA. pCD16-I (*8*) was used to probe a λ gt11 library (~5×10^5 recombinant phage) constructed with cDNA prepared from polyadenylated RNA of rIL-2 cultured human NK cells. cDNA were subcloned into Bluescript (Stratagene, La Jolla, California) and sequenced using the dideoxynucleotide chain termination technique with T3 and T7 primers and Sequenase (US Biochemical, Cleveland, Ohio). The termination site in pCD16-I at codon 234 was converted to Arg (AGG) by site-directed mutation (*17*) using an AAGACAAACATT*AGG*AGCT-CAACA oligonucleotide primer. This cDNA is referred to as CD16-I.AGG, and contains an additional Sac I site as a result of the change. Hybrids between CD16-I and CD16-II cDNA were constructed using standard techniques, and all were sequenced to ensure that the substitutions were successful.

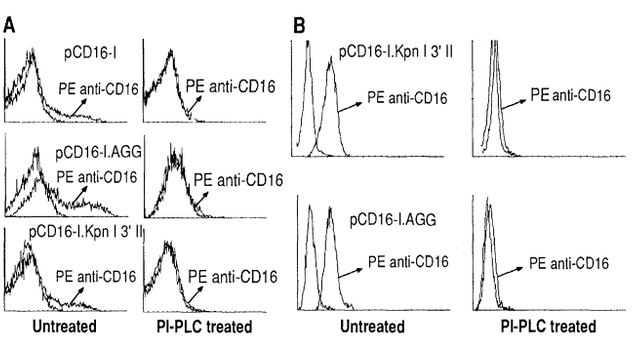

Fig. 2. Expression of CD16 cDNA in COS-7 (**A**) and Jurkat cells (**B**). cDNA were transferred into CDM7 or CDM8 and were transfected into COS-7 cells with DEAE-dextran (*18*). Transfected COS-7 cells were harvested after 48 to 72 hours and analyzed for expression. CD16-I.AGG in CDM7 was ligated to the p205L 18A mammalian expression vector and was used to transfect J.RT3-T3.5, a T cell receptor β chain mutant of Jurkat (*19*). Transfectants were selected in medium containing 400 μg/ml hygromycin B (Sigma, St. Louis, Missouri). A hybrid CD16 construction (designated pCD16-I.Kpn I-3′ II) contains CD16-I 5′ of the Kpn I site and CD16-II 3′ of the Kpn I site. The insert from pCD16-I.Kpn I-3′ II was subcloned into the Bam HI–Xba I site of the pTF-neo plasmid (*19*). This plasmid, designated pTFCD16-neo, was used to transfect J.RT3-T3.5 cells by electroporation, and transfectants were selected for growth in medium with geneticin (2 mg/ml, Sigma). Transfectants were incubated for 1 hour at 37°C in the presence or absence of *Bacillus thuringiensis* PI-PLC (0.1 U/ml, Immunotech, Marseille, France), washed, and stained with (A) PE-conjugated anti-Leu 11c (CD16) or a PE-conjugated IgG1 control MAb; (B) CLB FcGran I (CD16) MAb or an IgG2a control MAb, followed with PE-conjugated rat anti-mouse IgG2 MAb second step. Cells (5,000) were analyzed by flow cytometry (*5*). The *x*-axis represents fluorescence (4-decade log scale) and the *y*-axis the relative cell number. Histograms from cells stained with control MAb (nearest the ordinate) are superimposed. In the PI-PLC–treated COS-7 and Jurkat cells the anti-CD16–stained cells were indistinguishable from the control MAb-stained cells (that is, negative).

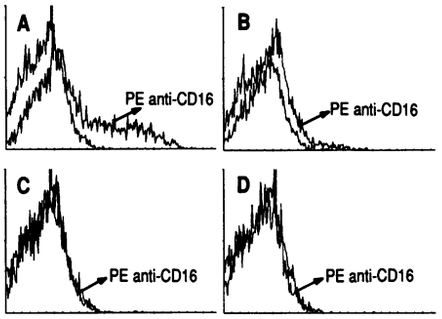

Fig. 3. Expression of pCD16 differing at residue 203. COS-7 cells were transfected with (**A** and **B**) pCD16-II.Ser^{203}, (**C**) pCD16-II, and (**D**) pCD16-I.AGG.Phe^{203}. Transfectants were harvested, incubated for 1 hour at 37°C in the absence (A, C, and D) or presence (B) of PI-PLC and analyzed for CD16 expression as described in Fig. 1. In C and D, the anti-CD16–stained cells were indistinguishable from the control MAb-stained cells (that is, negative).

with pCD16-I.AGG.Phe^{203}. Additional constructions in which the Bgl II–Kpn I fragment and Sal I–Kpn I fragment of CD16-II were subcloned into CD16-I.AGG (Fig. 1) also did not allow expression of CD16 on the surface of COS-7 cells. Thus, a single amino acid at codon 203 dictates membrane expression of CD16. Prior studies have implicated a COOH-terminal hydrophobic region in decay-accelerating factor as the signal sequence for PI-glycan linkage (*10*). Since both CD16-I and CD16-II have an identical hydrophobic segment in this region, this alone is insufficient to determine membrane anchoring. The ability of a single residue to influence membrane anchoring has been observed with Qa-2 (*11*).

Sequence analysis of CD16-II cDNA predicts that the polypeptide should be expressed as an integral transmembrane protein with a cytoplasmic domain. However, we were unable to obtain membrane expression of pCD16-II or chimeric constructions containing the Phe^{203} residue substitution into pCD16-I.AGG. Transfectants were analyzed with an extensive panel of MAb to exclude the possibility of epitope differences in COS-7 cells; CD16 transcripts were present in these cells as determined by Northern blot analysis. One explanation for lack of expression of pCD16-II is the possibility that a second protein is required for membrane expression. A similar situation exists for the tetramolecular high-affinity IgE receptor complex, where expression of the α subunit in COS-7 cells requires cotransfection with the γ subunit (*12*).

There are now several examples of membrane antigens that can be expressed as both integral transmembrane and PI-glycan–anchored proteins [CD58 (*13*), Qa-2 (*11*), and CD56 (N-CAM) (*14*)]. These different linkages are usually created by alternative splicing of a single gene, whereas CD16-I and CD16-II represent similar genes that differ in two important substitutions at codons 203 and 234. In the case of CD56, CD58, and Qa-2, an individual cell can coexpress both the PI-glycan and integral membrane proteins on the cell surface. In contrast, only the CD16-II polypeptide (36 to 38 kD) can be immunoprecipitated from ^{125}I-labeled NK cells, whereas granulocytes have only the smaller CD16-I (~30 kD) species (*3*). Since the pCD16-I.AGG mutant and pCD16-I.Kpn 1–3′ II hybrid cDNA possess the potential for the CD16-II transmembrane and cytoplasmic domains, as well as the Ser^{203} signal site for PI-glycan linkage, we compared CD16 expressed in COS-7 transfectants with the forms from granulocytes and NK cells. COS-7 transfectants were metabolically labeled with [^{35}S]Met, or surface labeled with ^{125}I, and CD16 was immunoprecipitated, deglycosylated with N-glycanase, and analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Both surface and metabolic labeling revealed a predominant ~30-kD CD16 polypeptide (Fig. 4). The absence of larger CD16 polypeptides from the metabolically labeled preparations indicated that the majority of the translated protein was efficiently cleaved by the protease involved with the addition of PI-glycan. Study of the PI-glycan–anchored trypanosome variant surface glycoprotein has demonstrated modification of the polypeptide within 1 min of translation, suggesting the addition of a preformed PI-glycan complex (*15*).

Only a single homologous murine gene, FcγRIIα, is expressed in murine NK cells and no PI-glycan–linked FcRγ has been identified (*16*). The regulated expression of CD16-I by granulocytes and CD16-II by NK cells in man is intriguing. Further studies into the regulatory elements controlling transcription of these genes may provide insights into this process.

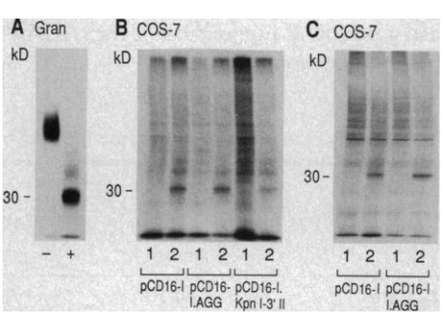

Fig. 4. Biochemical analysis of CD16 in transfectants. (**A**) ^{125}I-labeled granulocyte CD16 before (−) and after (+) deglycosylation. (**B**) Control immunoprecipitates (lane 1) and cell surface CD16 (lane 2) from transfected COS-7 cells. (**C**) Metabolically labeled, deglycosylated CD16 from transfected COS-7 cells. Cells were radiolabeled with ^{125}I (A and B) and lysed in tris-buffered saline (TBS, 50 m*M* tris, 0.15*M* NaCl, 0.02% NaN_3, *p*H 8.0) with 5 m*M* CHAPS [3(3-cholamidodropyl-dimethylammonio)-1-propane sulfonate] and protease inhibitors (*3, 20*). Transfected COS-7 cells (C) were metabolically labeled for 4 hours in Met- and Cys-free RPMI 1640 (Gibco, Grand Island, New York) that contained [^{35}S]Met and Cys (0.2 mCi/ml, Tran^{35}S-label, ICN, Irvine, California), 25 m*M* Hepes (*p*H 7.4), gentamicin, and dialyzed FCS (5%). Cells were lysed in TBS with 0.5% NP-40 and protease inhibitors [1 m*M* phenylmethyl–sulfonyl fluoride and aprotinin (20 KU/ml)] for 20 min at 4°C. Nuclei were removed by centrifugation and the cell lysate was precleared three times with 10 mg of packed Pansorbin (Calbiochem-Behring, San Diego, California) coated with saturating amounts of rabbit antiserum to mouse Ig. CD16 glycoproteins were immunoprecipitated from detergent lysates with anti-CD16–coated Pansorbin, treated with N-glycanase, denatured in sample buffer containing 2-mercaptoethanol (5%), and analyzed by SDS-PAGE using 10% acrylamide gel (*3, 20*).

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