A subunit common to an IgG Fc receptor and the T-cell receptor mediates assembly through different interactions

(Fcγ receptor type III/CD16/T-cell receptor/CD3/γ chain/protein assembly)

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ABSTRACT The γ subunit is a component of the Fcγ receptor of natural killer cells (FcγRIII or CD16), as well as the multimeric T-cell receptor/CD3 complex, and is required for assembly of both native receptors. The role of the γ subunit in human FcγRIIIA assembly differs from its role in T-cell receptor/CD3 complex assembly. The transmembrane domain of the FcγRIIIA α subunit forms noncovalent interactions with the comparable domain of the γ subunit and is sufficient for surface expression of the FcγRIIIA complex. In the absence of these interactions, sequences in the transmembrane domain of the FcγRIIIA α subunit signal its degradation. Leu-46, present in the transmembrane domain of the human γ subunit, is important for assembly with the FcγRIIIA α subunit. Substitution of this leucine with an isoleucine, as found in the mouse γ subunit, significantly reduces this interaction. In contrast, the mouse and human γ subunits interact with the pentameric T-cell receptor/CD3 complex, resulting in surface expression of this receptor.

Receptors for IgG immune complexes, the Fcγ receptors (FcγRs), are a heterogeneous family of membrane receptors on cells of the immune system that mediate a diverse array of cellular responses to antibody-antigen complexes (for review, see refs. 1–3). FcγRIIIA, the FcγR on natural killer (NK) cells, is an oligomeric complex that mediates antibody-dependent cellular cytotoxicity and NK-cell activation upon cross-linking with immune complexes (4). These responses are mediated through the stimulation of inositol phospholipid hydrolysis and increased intracellular calcium (5). FcγRIIIA is composed of at least three polypeptide chains: a ligand-recognition subunit, termed FcγRIIIα-γ, and the associated γ and ζ chains (6–11). FcγRIIIα-γ from human NK cells has been shown to exist in three molecular forms in association with γ2, ζ, or γζ chains (11). These associated chains are required for surface expression of the α subunit (8–10) and are presumed to be involved in signaling pathways, triggered by cross-linking of the α subunit by ligand. The γ and ζ subunits were initially identified as necessary components of other receptors. The γ chain is a component of the multimeric high-affinity Feε receptor for IgE (FcεRI) (12), and the ζ chain is a component of the T-cell receptor (TCR)/CD3 complex (13). The γ and ζ chains are required for surface receptor expression of FcεRI and TCR/CD3, respectively, and demonstrate significant sequence homology (14, 15). In TCR/CD3 assembly, the ζ chain plays a unique role in targeting pentameric complexes (TCR α and β chains/CD3 γ, δ, and ε chains) to the cell surface and sparing the incompletely assembled complex from degradation (16, 17). In this report, we describe results relevant to the mechanism by which the ζ chain mediates FcγRIIIA assembly and the sequence requirements for these assembly processes.

MATERIALS AND METHODS

Cell Culture and Antibodies. COS-7 cells were cultured in modified Eagle’s medium containing 10% (vol/vol) fetal calf serum. The peptide synthesized from the cytoplasmic domain of FcγRIIIA-α was used to raise the polyclonal antibody against the α chain. The γ and ζ chains were made by overexpressing their respective cDNA sequences in the bacterial system described by Studier and Moffatt (18). The complete coding sequences were cloned into the BamHI site of pET3xb to create a fusion protein with the T7 gene 10 protein and used to transform Escherichia coli BL21(DE3). The fusion protein was induced with isopropyl β-D-thiogalactoside and the overexpressed molecules were purified by SDS/PAGE. The eluted proteins were used to raise rabbit polyclonal antibodies.

DNA Constructions and DNA Transfection. Point mutants and hybrid molecules were constructed by PCR amplification or oligonucleotide-directed mutagenesis on single-stranded templates and cloned into the pCEX-V-3 expression vector (19). DNA was transfected into COS-7 cells by using calcium phosphate precipitation in the presence of 100 μM chloroquine followed by a 20% (vol/vol) glycerol shock.

Immunoprecipitation and Fluorescence-Activated Cell Sorter (FACS) Analysis. Transfected cells were permeabilized with digitonin (10 μg/ml) for 10 min on ice and iodinated with [125I] by 1,3,4,6-tetrachloro-3-α,6-α-diphenylglycouril (Iodo-Gen) (6). Cells were solubilized in lysis buffer containing 1% digitonin and 0.12% Triton X-100 (11). Cell lysates were sequentially incubated for 2 hr at 4°C with antibodies and then with protein A-Sepharose. Digestion with endoglycosidase H (endo H) was performed by incubating immunoprecipitated proteins for 16 hr at 37°C with 5 × 10−3 units of endo H in 0.1 M sodium phosphate (pH 6.1) containing 0.1% Triton X-100, 0.03% SDS, and 20 mM EDTA. Sepharose-bound immune complexes were eluted into sample buffer. FACS analysis was carried out using the anti-FcγIII monoclonal antibody (mAb) 3G8 or a nonbinding irrelevant mAb, B77, as described (10).

In Vitro Analysis. The cDNA sequences of α, γ, and ζ chains were cloned into pGEM-3Z and transcribed in vitro. These RNAs were translated in an in vitro translation reaction mixture containing 1 μg of the RNA in a nuclease-treated reticulocyte lysate (Promega) supplemented with dog pancreas microsomal membranes (kindly provided by Chris Nicchita, Rockefeller University, New York). Translation products were labeled with [35S]methionine and [35S]cysteine. After 1 hr of incubation at 30°C, a 10-fold volume of digitonin lysis buffer was added and the reaction mixture was immunoprecipitated with antibodies. The immune complexes were precipitated with protein A-Sepharose. Proteins were

Abbreviations: FcεR, Fcε receptor; FcγR, Fcγ receptor; TCR, T-cell receptor; NK, natural killer; FACS, fluorescence-activated cell sorter; endo H, endoglycosidase H; mAb, monoclonal antibody; ER, endoplasmic reticulum.
RESULTS

Previous results have demonstrated (8-10) that the γ or ζ subunit enhances surface expression of FcγRIIIA-α by as much as 50-fold. To determine the mechanism by which the ζ subunit mediates FcγRIIIA assembly, we transfected singly, and in combination, FcγRIIIA-α and γ and ζ chain cDNAs into COS cells and assayed for their surface expression. FcγRIIIA-α alone resulted in barely detectable surface expression; coexpression of α subunit with either γ or human ζ chain resulted in a 20-fold stimulation of surface expression of FcγRIIIA-α. Surface labeling and immunoprecipitation of these transfecteds demonstrated oligomeric complexes of α chain with homodimeric γ or ζ chain (Fig. 1 A and B). That γ and ζ chains were found as disulfide-linked dimers was demonstrated by analyzing the protein complexes immunoprecipitated from these transfecteds on nonreducing gels (data not shown). COS cells transfected with FcγRIIIA-α and γ and human ζ chain cDNAs formed three types of complexes on the cell surface: α chain with disulfide-linked γ, ζ, or γζ chain (Fig. 1C). These three complexes are similar to those observed on human NK cells (11), demonstrating that the interactions of α, γ, and ζ chains can be reconstituted by their coexpression in COS cells and do not appear to require other NK-cell-specific proteins.

The mechanism by which γ and ζ subunits facilitate surface expression of multimeric complexes containing α subunit was determined by characterizing the intracellular fate of FcγRIIIA-α in the presence or absence of γ and ζ chains. Pulse-chase labeling of the COS cells transfected with FcγRIIIA-α alone, shown in Fig. 2B, indicates that the α chain is degraded over the course of 6 hr. Endo H treatment of pulse-chase-labeled immunoprecipitates demonstrates that the α chain does not acquire resistance to this enzyme, implying the inability of the α subunit to reach the Golgi system. In contrast, pulse-chase studies of COS cells transfected with γ and ζ chain cDNAs demonstrate that these molecules are stable (data not shown) and are capable of reaching the cell surface in the absence of α chain (Fig. 1 A and B). Coexpression of α with γ or ζ subunit resulted in stable accumulation of α subunit, and the processed protein

acquired resistance to endo H (Fig. 2 C and D). FcγRIIIB, normally expressed as a glycosyl phosphatidylinositol anchored protein in neutrophils (6), is rapidly converted to an endo H-resistant molecule, indicating that its processing and movement to the Golgi are more rapid than FcγRIIIA (Fig. 2 A). These results indicate that γ and ζ chains can interfere with the degradation of the α chain and allow these complexes to reach the Golgi system. It is likely that this occurs through noncovalent interactions between the α chain and the γ or ζ chain in the endoplasmic reticulum (ER) as shown in Fig. 3. mRNAs for FcγRIIIA-α and γ and ζ chains, synthesized in vitro, were translated in a cell-free system in the presence of microsomes and immunoprecipitated with antibodies directed against each chain. These microsome preparations contain only ER membranes and are free of con-
taminating Golgi (22). The same complexes observed to form among α, γ, and ζ chains in vivo are also formed in vitro, indicating that only ER membranes are required for these interactions.

FcyRIIIA-α contains a signal that results in its degradation. We have shown (10) that deletion of the cytoplasmic domain of FcyRIIIA-α did not affect its degradation. A series of chimeric molecules were constructed with FcyRIIIA-α and γ and ζ chains to identify this degradation signal (Fig. 4A). When the transmembrane and cytoplasmic domains of the α chain were replaced with the comparable domains of the γ or ζ chain, stable accumulation and cell surface expression of these chimeric molecules were observed (ahZ or ayZ, respectively). The same result was obtained when the mouse FcyRII transmembrane and cytoplasmic domains were substituted for mouse FcyRIII-α sequences (R. L. Weinshank, and J.V.R., unpublished results). These experiments suggest that the 21-amino acid transmembrane sequence of the α chain contains a signal that determines its degradation. The interaction of FcyRIIIA-α with γ or ζ chain in the ER protects it from degradation and probably masks this signal. γ and ζ chains may also provide the appropriate signal for directing the α-chain-containing complex from the ER to the Golgi.

The sites of interaction between α and ζ chains could be mapped as a consequence of our previous observation that coexpression of the human ζ chain with an α chain in COS cells results in efficient surface expression, whereas the mouse ζ chain is 5–7% as efficient in its ability to function in the assembly of either human or mouse FcyRIIIA-α (10). Chimeric molecules constructed between human and mouse ζ chains were tested for their ability to enhance FcyRIIIA-α surface expression (Fig. 4A). The sequences of the ζ chain

### Figure 4

**A**  
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**B**  

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<th>Human ζ</th>
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<td>MIPAVVLILLLEQGMAA<strong>L</strong>EPPCFTIDAILFLYCVVIILYVPKIYQVKAATTSYKSDQVY</td>
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<td>MOKALPATAlAQDPTAPFPGPLDLQVCYLLGDLFLTYLYTVSRVALPWFRPSAEFPQDQMLY</td>
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**Fig. 4.** Mapping the domains of the ζ chain that are required for surface expression of FcyRIIIA-α. (A) Chimeric molecules and point mutations of the ζ chain. Point mutants and hybrid molecules were transfected singly or cotransfected with FcyRIIIA-α into COS-7 cells. Surface expression was determined by FACS analysis using mAb 3G8 (anti-FcyRIII) and expressed as a fraction of wild type (FcyRIIIA-α plus human ζ chain). Data were calculated as mean values from three or four experiments. The schematic structure of each molecule is shown, with the immunoglobulin-like extracellular domains indicated by semi-circles and the transmembrane domains as jagged lines. h, Human; Z, ζ; m, mouse. Letters in parentheses are substituted amino acids (single-letter code). (B) Sequence comparison of γ and ζ chains is shown for the region responsible for association with FcyRIIIA-α (NH2-terminal extracellular domain, transmembrane domain, and first 21 residues of the intracytoplasmic domain). Arrow indicates the Leu-46 in human, which is conserved among human γ and mouse γ chains but differs in mouse ζ chain and which influences the ability of ζ chain to interact with FcyRIIIA-α.
that confer this difference on the surface expression of $\alpha$ chain were mapped to the 9-amino acid extracellular domain, the transmembrane domain, and the first 21 amino acids of the cytoplasmic domain. The amino acid sequences of human and mouse $\zeta$ chains were compared to that of $\gamma$ chain (Fig. 4B) to identify sequences that are conserved among human $\zeta$ and $\gamma$ chains and divergent in mouse $\zeta$ chain. Such sequences could be responsible for this functional difference in Fc$\gamma$RIIIA-$\alpha$ interaction. Leu-46 of the human $\zeta$ chain is conserved in $\gamma$ chain at position 39 but is an isoleucine in mouse $\zeta$ chain. To determine the role of this difference in the ability of $\zeta$ chain to interact with $\alpha$ chain, single amino acid substitutions were made at this position in mouse and human $\zeta$ chains (Figs. 4A and 5). When Leu-46 was mutated to isoleucine in human $\zeta$ chain, the interaction with $\alpha$ chain was reduced by 65%. Similarly, mutation of Ile-46 to leucine in the mouse sequence led to a 5-fold stimulation in its ability to assemble with $\alpha$ chain (Figs. 4A and 5). Other amino acid differences between mouse and human $\zeta$ chains in the transmembrane region and the 21 residues of the cytoplasmic regions were tested for their role in $\alpha$-$\zeta$ chain interactions. No effect was observed in the efficiency of the surface expression of $\alpha$ chain when these single and double mutants were transfected with $\alpha$ chain cDNA (Fig. 4A). The Leu $\rightarrow$ Ile substitution did not affect the in vivo stability of $\zeta$ chain as determined in pulse-chase experiments; the mouse $\zeta$ protein was able to reach the cell surface as efficiently as its human counterpart (data not shown). Thus the difference in the efficiency of interaction of Fc$\gamma$RIIIA-$\alpha$ with human or mouse $\zeta$ chain results from the reduced ability of $\zeta$ chain to form a noncovalent complex with $\alpha$ chain in the ER, as can be seen from in vitro reconstitution experiments shown in Fig. 3. When mouse $\zeta$ mRNA was translated in a cell-free extract supplemented with microsomal membranes and $\alpha$ chain, a complex between these chains could not be detected, in contrast to the situation when human $\zeta$ chain was used.

$\gamma$ and $\zeta$ chains exist as disulfide-linked homo- and heterodimers (7, 11, 16, 20, 23) in all the receptor complexes in which they have been found. To determine if disulfide-linked dimerization of these subunits is essential for their ability to assemble with Fc$\gamma$RIIIA-$\alpha$, we mutated the cysteine at position 32 to a serine in the human $\zeta$ chain and constructed the homologous cysteine mutation (Cys-25 $\rightarrow$ Ser) in the $\gamma$ chain. These molecules were expressed on the cell surface but were incapable of disulfide-linked dimerization, as demonstrated on nonreducing gels (data not shown). There are two cysteines (Cys-25 and Cys-44) in a $\gamma$ chain. Mutation of Cys-44 to Ser did not affect disulfide-linked dimerization of $\gamma$ chains, indicating that Cys-25 is responsible for disulfide bond formation. These mutated $\gamma$ and human $\zeta$ chains were still able to associate with Fc$\gamma$RIIIA-$\alpha$ upon coexpression in COS transfectants, although surface expression of the complex was reduced by 40% for the $\zeta$ mutant and 10% for the $\gamma$ mutant (Fig. 5).

**DISCUSSION**

The ligand-binding subunits of low-affinity IgG Fc receptors, Fc$\gamma$RII and Fc$\gamma$RIII, differ in their ability to be efficiently expressed on the surface of heterologous cells. Mouse Fc$\gamma$RIII-$\alpha$ was $\approx$10% as efficient as Fc$\gamma$RII in its ability to be expressed on the surface of transfected cells (7, 10, 24). This modest level of surface expression for Fc$\gamma$RIII-$\alpha$ was achieved only after increasing the concentration of stable mRNA in the transfectants by the truncation of its 5' untranslated sequences (thereby deleting degradation signals) and increasing the concentration of transfected DNA. Surface expression of the $\alpha$ subunit could then be reproducibly detected in the absence of associated chains.

This decreased efficiency of Fc$\gamma$RIII-$\alpha$ compared to Fc$\gamma$RII expression results from its requirement for subunit interactions. Both the $\gamma$ subunit of FceRI and the $\zeta$ subunit of TCR/CD3 associate with Fc$\gamma$RIIIA-$\alpha$ (7–11). The transmembrane domain of the $\zeta$ subunit interacts directly with the comparable domain of Fc$\gamma$RIIIA-$\alpha$ in the ER and is sensitive to changes in specific amino acids in this domain. This role for $\zeta$ chain in assembly of Fc$\gamma$RIIIA-$\alpha$ is different than its role in TCR/CD3 assembly. Direct interaction of $\zeta$ chain with the ligand-binding subunit TCR$\alpha$ does not occur; rather a $\zeta$ chain interacts with the pentameric precursor $\alpha F_{\gamma} \beta \delta \varepsilon$ (16, 17). Both human and mouse $\zeta$ chains interact with the pentameric TCR/CD3 complex sparing it from lysosomal degradation, thus resulting in its surface expression. Despite the extensive sequence homology between mouse and human $\zeta$ chains, only the human $\zeta$ chain is capable of directly interacting with Fc$\gamma$RIIIA-$\alpha$, indicating that the amino acid difference Leu $\rightarrow$ Ile between human and mouse $\zeta$ molecules distinguishes one type of receptor assembly (Fc$\gamma$RIIIA) and another (TCR/CD3). Thus, in mouse NK cells, one type of Fc$\gamma$RIIIA complex, $\alpha F_{\gamma} \delta \varepsilon$, would be predicted to be absent, although complexes containing $\alpha$, $\gamma_2$, or $\alpha \gamma \zeta$ would still be capable of assembling.

The fate of the isolated ligand-binding subunits of Fc$\gamma$RIIIA or TCR/CD3 is similar. Both Fc$\gamma$RIIIA-$\alpha$ and TCR$\alpha$ contain signals in their transmembrane domains that result in their degradation in the ER (25, 26). These molecules do not acquire endo H-resistant glycosylation, and blocking the transport of these molecules from the ER to the Golgi with brefeldin A does not affect their fate (25 and data not shown). However, noncovalent interactions of an associated subunit with these sequences prevent their degradation. In this respect, the role of the $\gamma$ or $\zeta$ chain in Fc$\gamma$RIIIA assembly is similar to that of the CD3$\epsilon$ chain in TCR/CD3 assembly (26). The assembly of Fc$\gamma$RIIIA described here is likely to be shared by the tetrameric Fc receptor for IgE, FcERI. It has a ligand-binding $\alpha$ subunit that shares substantial sequence homology to Fc$\gamma$RIIIA-$\alpha$, particularly in its transmembrane domain (27, 28) and, for the human receptor, interacts directly with the $\gamma$ chain. From the results presented herein, we would expect that the interaction of the transmembrane domain of FceRI-$\alpha$ with $\gamma$ chain would be similar to the interaction of Fc$\gamma$RIIIA-$\alpha$ with $\gamma$ and human $\zeta$ chains.

The interactions between these ligand-binding subunits and their associated chains in the ER result from specific

**Fig. 5.** FACS analysis of COS cells transiently expressing the indicated mutants and Fc$\gamma$RIIIA-$\alpha$. $\gamma^*$ and $h^*$ indicate the $\gamma$ mutant (Cys-25 $\rightarrow$ Ser) and the human $\zeta$ mutant (Cys-32 $\rightarrow$ Ser), respectively. Control fluorescence (dotted line) was determined using a nonbinding mAb, B77. h, Human; z, $\zeta$; m, mouse. Letters in parentheses are substituted amino acids (single-letter code).
protein interactions, occurring in the lipid bilayer. Both the FcγRIIIA-α and the TCRα have charged amino acids in their transmembrane domain. These residues appear to play a critical role in the assembly of these subunits in the ER. Mutation of the Asp-222 residue in the transmembrane domain of FcγRIIIA-α decreases its susceptibility to degradation and reduces its ability to interact with γ and human ζ chains (10). Similar observations have been made for TCRα assembly with CD3ζ (26). In contrast to the role of the transmembrane domains in assembly, truncation of the cytoplasmic domains of either the α or the human ζ chain has a less dramatic effect on this process (10). In addition to the role of charged residues interrupting the hydrophobic stretch of amino acids spanning the bilayer, a surprising degree of specificity was observed in the hydrophobic interactions that occur in this domain. The Leu → Ile substitution in the transmembrane domain of ζ chain resulted in a significant decrease in its ability to interact with FcγRIIIA-α.

Thus the results presented herein indicate that a high degree of specificity is contained within the transmembrane domains of oligomeric receptor complexes. The role of this domain is considerably more dynamic and complex than previously thought. Rather than functioning as a passive hydrophobic nonspecific anchor through the lipid bilayer, this domain contains specific recognition signals for degradation, receptor assembly, and subcellular targeting.

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