

CHARACTERIZATION OF THE FAMILY OF DIMERS ASSOCIATED WITH Fc RECEPTORS (Fc ϵ RI and Fc γ RIII)

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The receptor for IgE (Fc ϵ RI) is a multimeric complex containing one α chain, one β chain with four transmembrane domains and one homodimer of disulfide-linked γ -chains. The Fc ϵ RI γ -chains form additional disulfide-linked dimers with the homologous ζ - and η -chains, as part of the TCR complex. The low affinity receptor for IgG (Fc γ RIII)² on NK cells is also associated with ζ -chains. Here we show that the γ -chain is expressed in NK cells both as a group of heterogeneous $\gamma\gamma$ homodimers and also as a heterodimer bound to ζ . Fc γ RIIIA is associated with three types of dimers $\zeta\zeta$, $\gamma\zeta$, and notably $\gamma\gamma$ as well. In fact, $\gamma\gamma$ appears to be the predominant species associating with Fc γ RIIIA. The surface expressed Fc ϵ RI also associates with the same group of heterogeneous $\gamma\gamma$ homodimers. We also show that there is no C-terminal posttranslational cleavage of γ occurring before its insertion into the plasma membrane as previously suggested. Thus, like the TCR, Fc γ RIIIA may form a variety of receptor isoforms, though at present we do not understand the functional implications of these structures.

The high affinity receptor for IgE is a tetrameric complex of noncovalently attached subunits ($\alpha\beta\gamma_2$), whose activation mediates a variety of functions, including the release of potent substances such as histamine, responsible for allergic reactions (1, 2). This receptor (Fc ϵ RI) is found exclusively on the surface of mast cells and basophils and transcripts for the α and β subunit have only been detected in these cells. However, the γ subunit message is also present in other hematopoietic cells that do not express Fc ϵ RI. The homodimer $\gamma\gamma$ is found in

mouse macrophages (3) where it associates with Fc γ RIII, a receptor with low affinity for IgG. Furthermore γ -chains are present in a mouse T cell line (CTLL) where they associate with ζ - and η -chains as part of the TCR complex, creating a rich structural diversity of homodimers and heterodimers whose function is at present unclear (4). Interestingly, homodimer of ζ -chains and an undefined ζ -chain-containing heterodimer also associate with Fc γ RIIIA (CD16) in human NK cells (5-7). In addition, cotransfection studies show that either γ and ζ can complement the surface expression of human Fc γ RIIIA (6, 8, 9). However, complementation for surface expression of receptors in transfection studies does not necessarily indicate what happens naturally. For example, human ζ can substitute for γ in promoting the surface expression of Fc ϵ RI (10). The ζ -chain being absent in mast cells and basophils, it is clear that an interaction between ζ and the other subunits of Fc ϵ RI does not occur naturally. Therefore it is important to demonstrate directly that these interactions do in fact occur in untransfected cells. In this study, we have characterized several types of dimers that associate with Fc γ RIIIA in human NK cells and identified a group of $\gamma\gamma$ homodimers in association with both Fc γ RIIIA and Fc ϵ RI.

MATERIALS AND METHODS

Cell lines and cell purifications. Rat basophilic leukemia cells (RBL-2H3) were grown as previously described (11). Human NK cells (LGL³) were purified from buffy coats of blood donated by several normal volunteers by sequential Ficoll/Hypaque gradients, adherence to plastic and nylon, Percoll gradients, and sheep E rosetting. (12). Flow cytometry of the resulting NK populations showed: >80% CD56⁺, <10% CD3⁺, $\approx$ 75% CD16⁺, $\approx$ 75% CD11b⁺, <2% Leu M3⁺. NK 3.3 cell line (a gift from Dr. J. Kornblut) (13), the YT cell line (14) and the preparation of basophil-enriched human leukocytes (15) have been described. CEM-CM3 and JURKAT cell lines were obtained from American Type Culture Collection (Rockville, MD).

Peptide synthesis and antibodies. Two short peptides corresponding to the cytoplasmic tail of human γ were synthesized. The sequences of the two peptides are as follows: CLSTRNQTETLTK (peptide 1: amino acids 68-80) and CKHEKPPQ (peptide 2: amino acids 80-86). An amino-terminal cysteine was added to the native sequence of each peptide. The synthetic peptides were made on the Milligen 9050 Pepsynthesizer with 9-fluorenylmethyloxycarbonyl chemistry, using the manufacturer's reagents and recommended chemistry cycles. Peptides were cleaved from the resin, precipitated, and extracted several times with anhydrous ether. Final purification was by preparative HPLC on a Waters C18 Delta-Pak (19 mm $\times$ 30 cm, 15 μ m particle, 300 μ m pore size), using 0.1% trifluoroacetic acid in water as the starting solvent followed by 0 to 50% acetonitrile gradient in 0.1% trifluoroacetic acid. Amino acid composition, amino acid sequence analysis, and analytical reverse phase HPLC were performed to confirm peptide sequence and purity. The cross-linking

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² We are following a new nomenclature on FcR proposed by J. V. Ravetch and J. P. Kinet (see Ref. 1). This nomenclature incorporates the recommendations of previous nomenclature committees and, where possible, tries to conform to the standards established by the WHO. Fc γ RIII is encoded by a single gene in the mouse (previously named Fc γ RIIIa, Fc γ RIIIa, or Fc γ RIII α) and by two genes in the human, Fc γ RIIIA (previously named Fc γ RIII-2 or Fc γ RIII-TM; this gene corresponds to one form of CD16 and is expressed in NK cells, macrophages, some T cells and is inducible in monocytes) and Fc γ RIII (previously named Fc γ RIII-1 or Fc γ RIII-PIG; this gene corresponds to the other form of CD16 and is expressed only in neutrophils, its protein product being anchored in the plasma membrane by a glycosyl-phosphatidylinositol moiety).

³ Abbreviation used in this paper: LGL, large granular lymphocytes.

agent sulfo-m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (Pierce Chemical Co., Rockford, IL) was used to couple the sulfhydryl group of the cysteine residue to chicken serum albumin. Each of the coupled peptides were then used for immunization of three different rabbits. The rabbit sera were tested for anti- γ activity by immunoprecipitation of ^{125}I -IgE-receptor complexes. Furthermore, the specificity of the antisera was tested by immunoblotting. The serum 581 (antipeptide 1) and the serum 667 (antipeptide 2) were selected for this study. As shown in a previous study (4) the serum 581 did not show any cross-reactivity with ζ . In addition, the polyclonal antipeptide antibodies 581 were affinity purified using a column of peptide 1 bound to Epoxy activated-Sepharose 6B beads (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ). Rabbit anti- ζ antiserum 387 was kindly provided by R. Klausner (National Institutes of Health, Bethesda, MD) (16). Murine mAb anti-CD16 (3G8) (17) was purchased from Medarex Inc. (West Lebanon, NH). Mouse monoclonal antidi-nitrophenyl IgE (18) was prepared, purified, and iodinated as previously described (11).

Northern blotting. Total cytoplasmic RNA from the different cell lines and from LGL cells was extracted as described and Northern blotting was performed following a standard protocol (19, 20) except for the use of Nytran membranes (Schleicher & Schuell Inc., Keene, NH). The same blots were successively hybridized with the following complementary DNA probes: the *Xba*I-*Nde*I fragment of the human Fc ϵ RI γ cDNA (15) the *Xba*I-*Eco*RI fragment of the human TCR ζ cDNA (a gift from R. Klausner) (21), the *Eco*RI-*Bam*HI fragment of human Fc γ RIIIA cDNA (a gift from J. Ravetch) (22).

Immunoprecipitations. The preparation of IgE-receptor complexes has already been described (23). Briefly, the IgE was bound to the surface of RBL-2H3 cells, the cells were washed and lysed in a borate saline buffer containing 10 mM of 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate and protease inhibitors in conditions of minimal receptor dissociation. The postnuclear extract was then incubated with rabbit anti-mouse IgE for 2 h at 4°C and the immune complexes were precipitated with protein A-Sepharose beads (Pharmacia).

Total cellular γ -chain from RBL cells and from human NK cells was immunoprecipitated directly from 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate lysates by the purified rabbit antipeptide 1 and protein A beads. The ζ -chains from human NK cells were similarly immunoprecipitated. The ζ -CD16 complexes and γ -CD16 complexes from human NK cells were solubilized with a digitonin lysis buffer (20 mM Tris-HCl, pH 7.4, containing 1% digitonin, 0.12% Triton, 150 mM NaCl, 10 mM iodoacetamide, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, 10 mM PMSF) and immunoprecipitated with anti-CD16 mAb 3G8 and protein G Sepharose beads (Pharmacia). Iodoacetamide (10 mM) was included in all buffers to prevent nonphysiologic disulfide formation.

Electrophoresis and immunoblotting. Sepharose-bound immune complexes were eluted into boiling nonreducing sample buffer. The eluted proteins were separated by two-dimensional nonreducing/reducing SDS-PAGE as described (24). The separated proteins were electrophoretically transferred onto Immobilon P membrane (Millipore, Bedford, MA). The membranes were incubated at room temperature for 1 h in PBS, pH 7.4, containing 5% nonfat milk and then for 2 h in PBS, 2.5% milk containing 10 $\mu\text{g}/\text{ml}$ of anti-Fc ϵ RI- γ antibodies or of nonspecific rabbit IgG as a control, or 1/200 dilution of the anti- ζ serum. After removal of unbound antibodies, the membranes were incubated for 1 h in PBS, 2.5% milk, and 0.5 $\mu\text{Ci}/\text{ml}$ of ^{125}I -labeled protein A (ICN, Irvine, CA), then washed for 1 h in PBS, 0.1% Triton X-100 before being subjected to autoradiography.

RESULTS

Expression of Fc ϵ RI γ -chains in various human cells. Total cytoplasmic RNA was prepared from purified human NK cells (LGL), from basophil-enriched human leukocytes and from two NK cell lines (NK 3.3 and YT) and two T cell lines (Jurkat and CEM-CM3). Northern blot analysis (Fig. 1) using DNA probes specific for human CD16, human Fc ϵ RI γ -chain, and human TCR- ζ chain reveals different patterns of expression for these three transcripts. As expected, the two T cell lines express the TCR ζ message but not the CD16 message. CEM-CM3 but not Jurkat is positive for Fc ϵ RI γ . This shows that human T cells are also capable of expressing γ transcripts in the absence of Fc γ RIII, similarly to what we observed in a mouse cytotoxic T cell line. Confirming previous

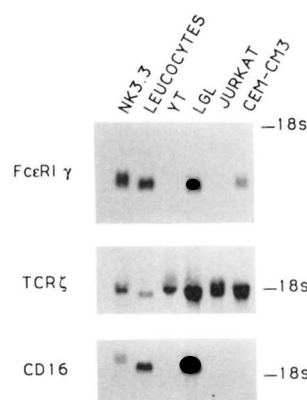


Figure 1. Northern blot analysis of Fc ϵ RI γ -chain, TCR- ζ , and CD16 mRNA levels in NK3.3 cell line, basophil-enriched leukocytes, YT cells, LGL cells, Jurkat cells, and CEM-CM3 cells. The migration of 18 S ribosomal RNA is indicated. The migration of lane 1 was slightly slower presumably as a consequence of a smiling artifact.

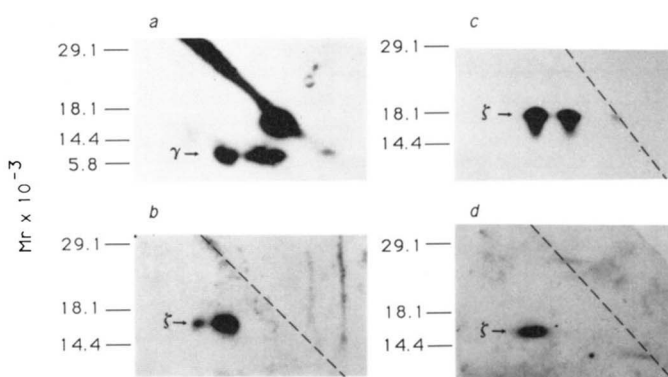


Figure 2. Expression and dimerization of γ and ζ in human NK cells (a-c) and YT cells (d). Cell lysates were immunoprecipitated using anti- γ antibodies (a and b) or anti- ζ peptide serum (c and d). The immunoprecipitates were resolved by two-dimensional nonreducing/reducing SDS-PAGE (first and second dimension 14% acrylamide) and immunoblotted using anti- γ antibodies (a) or the anti- ζ peptide serum (b-d). Our interpretation of the peptide seen in this figure is as follows. In a, peptides below the diagonal falling from 27 kDa: $\gamma\zeta$ heterodimer; falling from 24–20–17 kDa: $\gamma\gamma$ homodimers. b, c, and d, peptides below the diagonal falling from 32 kDa: $\zeta\zeta$ homodimer; falling from 27 kDa: $\gamma\zeta$ heterodimer.

reports, human LGL cells express CD16 and TCR ζ -chain transcripts. The same transcripts are also seen in NK 3.3 cells. In addition, both LGL and NK 3.3 cells express Fc ϵ RI γ message. This contrasts with the NK-like YT cell line that is positive for TCR ζ but negative for both CD16 and γ .

Characterization of $\zeta\zeta$, $\gamma\gamma$, and $\gamma\zeta$ dimers in human NK cells. We next investigated the expression and dimerization of γ and ζ proteins in human NK cells. After solubilization of LGL cells, γ -chains were immunoprecipitated from total cellular lysates with anti- γ (antipeptide 1). Then the anti- γ immunoprecipitates were resolved by two-dimensional nonreducing/reducing SDS gels and immunoblotted with anti- γ (Fig. 2a). The γ -chain is strikingly heterogeneous in NK cells. Multiple 8 kDa γ species were identified below the diagonal and two types of γ monomer of 8 and 16 kDa were consistently seen on the diagonal (Fig. 2a). Although the polypeptides seen below the diagonal do not have discrete mobilities, they could potentially correspond to γ -containing peptides migrating at approximately 27, 24, 20, and 17 kDa in the nonreduced dimension. This group of γ -containing peptides associates also with the cell surface expressed Fc ϵ RI from RBL cells that lack ζ chains (see Fig 3a and below). We

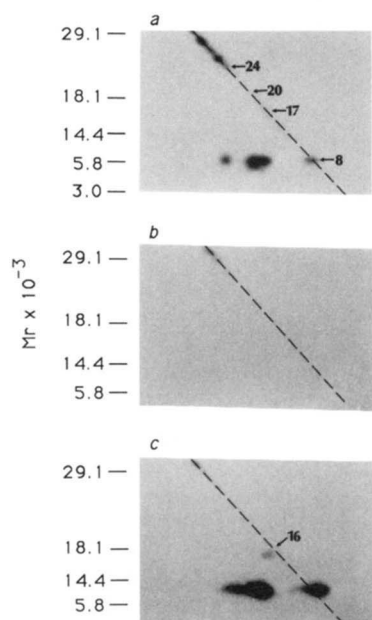


Figure 3. Characterization of surface expressed and intracellular γ -chains in RBL-2H3 cells by western blotting. IgE-receptor complexes from the cell surface (a) and γ -chains from total cellular lysates (b and c) were immunoprecipitated with anti-IgE and anti- γ antibodies, respectively. The immunoprecipitates were analyzed and immunoblotted as in Figure 2 with anti- γ antibodies (a and c) or nonspecific rabbit IgG (b). The arrows indicate the m.w. of the relevant peptides in the nonreduced dimension. Anti- ζ immunoblots of the same immunoprecipitate did not reveal any peptides.

identified the 27-kDa peptide as being $\gamma\zeta$ heterodimers by immunoblotting immunoprecipitates of LGL γ -chains with anti- ζ . Under these conditions a prominent 16-kDa ζ species is seen below the diagonal falling from 27 kDa in the nonreducing dimension (Fig. 2b). In addition, a small amount of 32-kDa $\zeta\zeta$ homodimers appears to be precipitated via the γ -chain. Inasmuch as anti- γ does not cross-react with ζ , it is possible that the coprecipitation of $\zeta\zeta$ with anti- γ is the result of the coassociation of $\zeta\zeta$ and γ -containing dimers with CD16 (see below). As expected, the same ζ species corresponding to $\zeta\zeta$ and $\gamma\zeta$ are also seen when anti- ζ immunoprecipitates from LGL cells are immunoblotted with anti- ζ (Fig. 2c). To support the contention that the 27 kDa represents $\gamma\zeta$ heterodimer, we analyzed YT cells that express ζ but not γ and observed that the heterodimer $\gamma\zeta$ was lacking in these cells (Fig. 2d). Furthermore, the association of γ with ζ was confirmed in anti- γ immunoblots of anti- ζ immunoprecipitates (not shown). From these experiments it can be concluded that $\zeta\zeta$ homodimers (32 kDa) and $\gamma\zeta$ heterodimers (27 kDa) are present in human NK cells. We do not understand at present how the group of γ peptides (ranging from 24 to 17 kDa) arise. Obviously, it is difficult to exclude that the group of γ peptides comes from other associated molecules. However the presence of the same peptides in RBL cells (see below) makes it unlikely. Rather, we think that it is more likely that the heterogeneity of the γ peptides is generated from a posttranslational modification of the $\gamma\gamma$ homodimers (discussed below).

Characterization of γ monomers and $\gamma\gamma$ homodimers in RBL cells. To determine whether the different species of γ observed in Figure 2a are specific for human NK cells, we analyzed the 2D pattern of γ -chains in whole cell lysates of RBL cells that do not express ζ -chains.

Interestingly the same γ peptides except one, are also seen in RBL cells (Fig. 3c). The 8- and 16-kDa γ monomers are clearly seen on the diagonal although in different proportion and multiple 8-kDa γ -containing peptides are detected below the diagonal falling from M_r ranging from 24000 to 17000 in the nonreducing dimension (see also Fig. 3a). However, as predicted the 27-kDa γ -containing peptide corresponding to the $\gamma\zeta$ heterodimer in human NK cells, is absent in RBL cells.

We next tested whether these γ peptides were associated with cell surface Fc ϵ RI. To selectively purify cell surface receptors, RBL cells were bound with IgE before solubilization. The IgE-receptor complexes were precipitated with anti-IgE, resolved by two-dimensional gel and immunoblotted with anti- γ (Fig. 3a). Four distinct 8-kDa peptides were identified: the 8-kDa γ monomer on the diagonal and three 8-kDa γ -containing peptides that correspond to 24, 20, and 17 kDa nonreduced dimers. The 16-kDa γ monomer (see Fig. 3c), observed in larger amount in LGL cells (see Fig. 2a), could represent the unprocessed monomeric γ -chain before cleavage of the leader peptide. If so, it would explain why this peptide does not associate with the cell surface receptors Fc ϵ RI and CD16 (see below). Further analysis of the 24-, 20-, and 17-kDa γ -containing peptides indicate that they do indeed correspond to different species of $\gamma\gamma$ homodimers. These peptides but no additional peptides are seen below the diagonal after immunoprecipitation of γ -chains from metabolically labeled cells with [^{35} S]cysteine (not shown). Therefore these γ -containing peptides do not correspond to undefined heterodimers but to different forms of $\gamma\gamma$ homodimers.

Association of $\zeta\zeta$, $\gamma\zeta$, and $\gamma\gamma$ dimers with CD16 in human NK cells. To determine if the different dimers observed in NK cells are associated with CD16, anti-CD16 immunoprecipitates from human NK cells were analyzed by two-dimensional gel, transferred to Nytran membranes, and immunoblotted with anti- γ (Fig. 4a) and with anti- ζ (Fig. 4b). The anti- γ immunoblot reveals at least two 8-kDa γ species. One clearly corresponds to the 27-

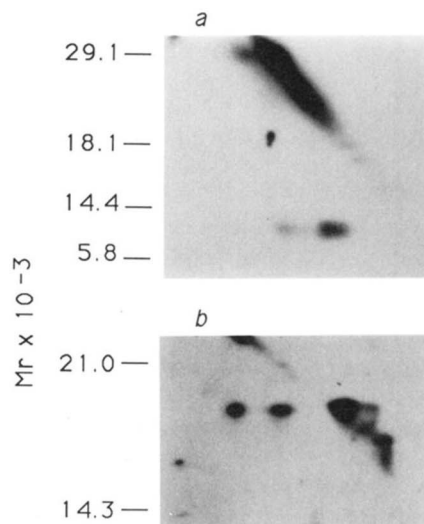


Figure 4. Identification of γ and ζ family dimers by Western blotting and demonstration of their association with Fc γ RIIIA (CD16) in human NK cells. Human NK cells were lysed in digitonin buffer and the cell lysates were immunoprecipitated with the mAb anti-CD16 3G8. The immunoprecipitates were then analyzed in two-dimensional gels and immunoblotted with anti- γ (a) or anti- ζ (b).

kDa $\gamma\zeta$ heterodimer. The other species (not well separated on this gel) correspond to 17- to 24-kDa $\gamma\gamma$ homodimers. Repetitive quantitative analysis of the counts with a radioanalytic imaging system (AMBIS) shows that 76% of the counts associated with γ -chains are part of $\gamma\gamma$ homodimers. Since only one γ -chain is represented in the $\gamma\zeta$ heterodimer, it can be calculated that the ratio of $\gamma\gamma/\gamma\zeta$ is 1.58. Obviously this calculation assumes that there is no preferential dissociation from CD16 of either one of the dimers. The anti- ζ immunoblot clearly shows that CD16 associate with $\zeta\zeta$ homodimers and $\gamma\zeta$ heterodimers. The intense spot seen on the right of the diagonal would correspond to monomeric ζ . This species is also evident in Figure 2c albeit much less intense. We do not understand the nature of the monomeric ζ (and γ) species but cannot exclude that it occurs because of inadvertent cleavage in vitro of the disulfide bond. It is possible however that monomeric ζ and γ associate with the surface expressed receptors (see also Fig. 3a). These results demonstrate unequivocally that CD16 in human NK cells associate with $\gamma\gamma$, $\gamma\zeta$, and $\zeta\zeta$ dimers with a preference for the $\gamma\gamma$ homodimer.

Absence of C-terminal cleavage of γ -chains. Biosynthetic dual labeling of RBL cells using [^{3} H]histidine and [^{35} S] cysteine indicated that the Fc ϵ RI γ -subunit purified from the plasma membrane did not incorporate [^{3} H]histidine (25). However, the γ cDNA sequence predicts a histidine residue close to the C-terminus. To explain this apparent discrepancy, we speculated that a posttranslational processing could cleave the C-terminus of the γ before its insertion in the plasma membrane. The sequence of one tryptic peptide of γ purified from the plasma membrane ended with the lysine residue that precedes the histidine residue predicted by the γ cDNA sequence. Therefore this lysine residue—the seventh residue from the C-terminus—was proposed to be the site for the posttranslational cleavage (25). To assess this possibility, cell surface IgE-receptor complexes and whole cells γ -chains were immunoprecipitated with anti-IgE and antipeptide 1, respectively, and analyzed by two-dimensional gels for immunoblotting with an antipeptide serum against the C-terminal sequence of γ (antipeptide 2). It is clear that the various $\gamma\gamma$ homodimers and the 8-kDa γ monomer react with this antibody regardless of their cell surface (Fig. 5a) or intracellular (Fig. 5b) localization. Therefore there is no C-terminal processing of γ -chains.

DISCUSSION

Previous reports have demonstrated the presence of TCR ζ -chains in human NK cells and the association of $\zeta\zeta$ homodimers with Fc γ RIIIA (6, 7). An additional heterodimer, ζ -p12 was also detected in association with Fc γ RIIIA (7) but was not fully characterized. Our results establish unequivocally that three types of dimers, $\zeta\zeta$, $\zeta\gamma$, and $\gamma\gamma$ associate with Fc γ RIIIA in human NK cells. In addition, we show that the association of $\gamma\gamma$ with Fc γ RIIIA seems to be preferential when compared with $\gamma\zeta$ and $\zeta\zeta$. Furthermore we describe a diversity of $\gamma\gamma$ homodimers in association with both Fc γ RIIIA and Fc ϵ RI. When analyzed in two-dimensional gel, these $\gamma\gamma$ homodimers show different apparent M_r in nonreducing conditions but generate monomers of identical M_r after reduction. This two-dimensional pattern could be due to fatty acids attached to γ -chains. We have previously

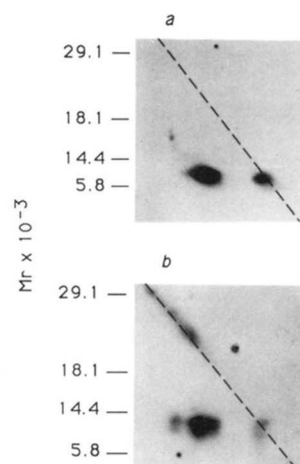


Figure 5. Assessment of the presence of the C-terminal end of γ -chains at the cell surface by Western blotting with an antipeptide against that region. Cell surface IgE-receptor complexes (a) and γ -chains from total cellular lysates (b) were prepared and analyzed as in Figure 3 before being immunoblotted with antipeptide 2, specific for the last seven amino acid residues of the γ -chain.

reported that the subunits of Fc ϵ RI contain ester-linked fatty acids (26). In particular, the β and γ associate covalently with palmitate. Recent studies on viral glycoproteins indicate that covalently bound acyl chains can be released by the action of reducing agents (27, 28). This would be consistent with our observation. The rationale is that a thioester linkage to cysteine residues could mediate palmitate attachment. If so, palmitoylation could generate different forms of homodimers but after reduction and release of the fatty acids, only one form of γ monomer would be observed. As discussed previously (26), the incorporation of radioactive fatty acids into the γ -chain is so small that a careful study of this hypothesis by using biosynthetic incorporation is impractical. Therefore, to test this hypothesis we have chosen to analyze whether mutation of the relevant cysteine of γ could change the mobility of the $\gamma\gamma$ homodimer without affecting the mobility of the γ monomer. In that case, these mutants will allow us to test also if these modifications are important in the functioning of γ .

Finally, we have demonstrated by direct analysis that there is no C-terminal processing of γ -chains as we had initially proposed (25). This suggestion was made to explain a reproducible lack of incorporation of [^{3} H]histidine into surface-expressed γ -chains although its amino acid sequence shows an histidine residue at the C-terminal end. This was a reasonable assumption because this kind of processing has been observed for other membrane proteins. It clearly does not happen in γ -chains. Therefore it is likely that the efficiency of incorporation of [^{3} H]histidine was too low in our experimental conditions.

The γ -, ζ -, and η -chains form a family of dimers, some of which associate with at least three different receptors: Fc ϵ RI, Fc γ RIII, and TCR. Future studies on structure-function relationship will be necessary to understand the basis for this structural diversity. It will be of great interest to assess whether the different dimers correspond to different coupling structures capable of mediating specific effector functions.

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