Alternative Endocytic Pathway for Immunoglobulin A Fc Receptors (CD89) Depends on the Lack of FcRγ Association and Protects against Degradation of Bound Ligand*

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IgA is the most abundant immunoglobulin in mucosal areas but is only the second most common antibody isotype in serum because it is catabolized faster than IgG. IgA exists in monomeric and polymeric forms that function through receptors expressed on effector cells. Here, we show that IgA Fc receptor(s) (FcR) are expressed with or without the γ chain on monocytes and neutrophils. γ-less FcR represent a significant fraction of surface FcR molecules even on cells overexpressing the γ chain. The FcR-γ2 association is up-regulated by phorbol esters and interferon-γ. To characterize γ-less FcR functionally, we generated mast cell transfectants expressing wild-type human FcR or a receptor with a point mutation (Arg → Leu at position 209) which was unable to associate with the γ chain. Mutant γ-less FcR bound monomeric and polymeric human IgA1 or IgA2 but failed to induce exocytosis after receptor clustering. The two types of transfectant showed similar kinetics of FcR-mediated endocytosis; however, the endocytosis pathways of the two types of receptor differed. Whereas mutant FcR were localized mainly in early endosomes, those containing FcR-γ2 were found in endo-lysosomal compartments. Mutant γ-less FcR recycled the internalized IgA towards the cell surface and protected against IgA degradation. Cells expressing the two forms of FcR, associated or unassociated with γ chains, may thus have differential functions either by degrading IgA antibody complexes or by recycling serum IgA.

In humans, IgA is found in the systemic and mucosal compartments; it is the second most common antibody class in blood and the major immunoglobulin at mucosal surfaces (1, 2). More IgA is produced daily than all of the other immunoglobulin classes together (3). In serum, IgA is mainly monomeric and has a half-life around five times shorter than that of IgG because of its fast catabolism (2, 4). Although the implications of secretory IgA in host defenses are well established (2), much less is known about the antibody-mediated functions of serum IgA in human blood. Serum IgA has been considered an anti-inflammatory isotype capable of inhibiting several functions mediated by other isotypes including inhibition of IgG phagocytosis, bactericidal activity, oxidative burst, and cytokine release (5–10). The molecular basis of these inhibitory functions is poorly understood; however, IgA-immune complexes can trigger effector cells after aggregation of IgA Fc receptor(s) (FcR, 1 CD89), resulting in various immune effector functions such as phagocytosis, oxidative burst, and cytokine release (11–13).

FcR are expressed on myeloid cells as heterogeneously glycosylated type I transmembrane proteins that can bind both IgA1 and IgA2 isotypes at the boundary between the C2 and C3 domains (14–18). Polymeric IgA binds more efficiently to FcR than does monomeric IgA (19, 20). FcR exist as at least two isoforms (a.1 and a.2) differing by a deletion in their extracellular domains and expressed alternatively on monocytes and alveolar macrophages (21). Several other splice variants, the corresponding native proteins of which have not been identified, have also been reported (21–25). FcR are associated with the disulfide-linked FcR γ chain homodimer (26–28). This interaction is resistant to treatment with Nonidet P-40 detergent, which contrasts with the dissociation of γ chains from FcERI or FcγRI in certain detergents (26, 29, 30). This strong interaction can be explained by the presence of two oppositely charged residues (Arg/asparagine) in the transmembrane domain of the FcR and γ chain, respectively (28). The γ chain contains a common immunoreceptor tyrosine-based activation motif in its cytoplasmic tail. Recently, it has been shown that signaling through FcRγ2 involves several tyrosine kinases including lyn, syk, and Btk (31, 32). Recruitment and phosphorylation of syk and Btk were modulated by stimulation with interferon-γ (IFN-γ) and/or phorbol esters, indicating that activation of tyrosine kinases through FcR depends on the priming state of the cell (32).

FcR without signaling motifs in their cytoplasmic tails are associated with specialized subunits, such as v or β chains, and depend on their specific retention motifs to be fully expressed on the cell surface (33). In the absence of the γ chain they are degraded rapidly in the endoplasmic reticulum as in the case of FcγRIII (34). One remarkable feature of FcR is that these receptors can be expressed fully at the surface of COS cells after transfection, without the signaling γ subunits (16). Despite the role of the γ chain in downstream FcR signaling (28),

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1 The abbreviations used are: FcR, Fc receptor(s); Ab, antibody(ies); mAb, monoclonal antibody(ies); PE, phycoerythrin; PAGE, polyacrylamide gel electrophoresis; RAM, rabbit anti-mouse Ig; GAM, goat antibody specific for mouse Ig; GAR, goat anti-rabbit Ig; FITC, fluorescein isothiocyanate; PMCA, phorbol 12-myristate 13-acetate; IFN-γ, interferon-γ GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; RBL, rat basophilic leukemia; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter.

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FIG. 1. Lack of colocalization between γ chain and some FcR within intracellular vesicles after short term endocytosis. PMA-treated U937 cells preincubated with human IgG (10 mg/ml) to block FcγR were incubated with anti-FcγR A77 F(ab’)_2 fragments plus GAM coupled to FITC on ice as described under “Experimental Procedures.” After FcγR staining, cells were incubated at 37 °C for 3 min, fixed, permeabilized, and stained with anti-γ chain polyclonal Ab plus GAR coupled to Texas Red. Cells were observed under a confocal microscope and optically sectioned at 1.5-μm intervals. A representative medial section of the horizontal slices is shown. No staining was observed when FITC-labeled secondary Ab or irrelevant IgG1 was used.

we wondered whether the FcγR could exist and function as receptors when unassociated with the γ chain (γ-less FcγR) on myeloid cells. We have identified significant amounts of γ-less FcγR in several cell types, including monocytes, neutrophils, and transfected cells overexpressing the γ chain. γ-less FcγR and FcγR-γ2 are expressed on the same cells, and this constitutes the basis for differential endocytosis pathways of IgA, in which γ-less receptors recycle IgA toward the cell surface whereas FcγR-γ2 undergo endo-lysosomal sorting for IgA degradation.

EXPERIMENTAL PROCEDURES

Antibodies—The following mouse mAb were used: A59 (IgG1κ), A77 (IgG1κ) mAb specific for FcγR (35), an irrelevant IgG1κ control mAb (clone 7.1 anti-glutathione S-transferase protein). My43 anti-FcγR mAb (IgM) was a gift from Dr. L. Shen, Dartmouth Medical School, Lebanon, NH (36). Phycoerythrin-labeled anti-FcγR A59 (A59-PE) was purchased from PharMingen (San Diego). 4D8 anti-FcγR chain mAb (IgG2bκ) was a gift from Drs. D. Presky and J. Kochan, Hoffman-La Roche, Nutley, NJ (37). Complete digestion and F(ab’)_2 purity were verified by SDS-PAGE. Rabbit anti-mouse Ig (RAM) antibodies were obtained from rabbits immunized with an IgG1 (clone A59). F(ab’)_2 fragments of A59, A77, and IgG1κ and RAM IgF fractions were prepared by pepsin digestion (Sigma) as described previously (38) and purified on DEAE columns. Rabbit antiserum specific for the Fcγ-γ chain was a gift from Dr. J. P. Kinet, Harvard Medical School, Boston (39). Fluorescein isothiocyanate (FITC)-conjugated goat Ab specific for mouse (GAM) and rabbit IgG (GAR) and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Southern Biotechnology Associates (Birmingham, AL). IgG myeloma proteins were purified as described previously (19). Monomeric and polymeric IgA preparations (>98% pure) were biotinylated.

Cells—The human monocytic cell line U937 was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Polymorphonuclear cells and mononuclear cells were isolated from whole blood by Ficoll-Hypaque (Amersham Pharmacia Biotech) gradient centrifugation. Granulocytes were purified from red cell pellets by dextran sedimentation as described previously (15). Enriched monocyte populations (60–80% pure) were obtained by subjecting mononuclear cells to rosette formation with 2-aminoethylisothiouronium bromide-treated SRBC, and nonrosetting cells were submitted to plastic adhesion as described in Ref. 19. In some experiments, cells were cultured for 18 h with 10^{-7} M PMA (Sigma) (15), 50 units/ml human recombinant IFN-γ (Genzyme, Cambridge, MA), 5 μg/ml ionomycin (Calbiochem, San Diego), 100 μM human recombinant GM-CSF (Sandoz AG, Basel, Switzerland), or 50 units/ml interleukin (IL)-1α (Rhône-Poulenc Santé, Vitry, France). Rat basophilic leukemia cells (RBL-2H3) (40) were transfected with human FcγR and/or human γ chain and were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, 1.5 μg/ml puromycin (Sigma) and/or 1.0 mg/ml of G418 (Life Technologies, Inc.).

Constructions, Expression Vectors, and Transfection—Human FcγR containing the R209L mutation was constructed by amplifying base pairs 591–891 of a previously described FcγR a.1 cDNA (21). The sense primer included the BanII restriction site at position 601 and introduced the R209L mutation (at base pairs 688–690, with CTG replacing CCC). The amplified fragment was ligated to the remainder of the terminal cDNA via the BanII restriction site. The construct was checked by sequencing as described in Ref. 21. The FcγR a.1, FcγR (R209L), and human γ chain (41) were subcloned into pSRNeo (kindly provided by Dr. J. Di Santo, INSERM U429) a modified version of the pcDL-SRA promoter-based expression vector (42). RBL-2H3 cells were first transfected with 30 μg of DNA by electroporation at 250 V and 1,050 microfarads using an Easyjet™ apparatus (Eurogentec, Seraing, Belgium), then grown under 1 ng/ml β-erg, 50 μg/ml puromycin (Sigma) and/or 1 μg/ml of polyclonal rabbit anti-γ chain mAb (nonspecific counts were always <1% of total cpm) for 1–2 days. By 4-day culture, more than 80% of the cells expressed human γ chain, determined by immunoblotting. The γ chain+ human FcγR-expressing clone was chosen and cotransfected with pSRNeo-human γ-κ chain (30 μg) and pSR-Puro (4 μg) (43).

Cell Idilination, Immunoprecipitation, and Immunoblotting—Cell surface iodination with Na^{125}I (1 mCi; Amersham Pharmacia Biotech) was carried out by the lactoperoxidase method (44). For immunoprecipitation of FcγR, cells (10^{5}/ml) were lysed for 30 min at 4 °C in PBS containing 1% digitonin (Aldrich), 0.02% sodium azide, 1 μM phentolylmalononitrile, and 5 mM iodoacetamide, and 1 μM phenylmethylsulfonyl fluoride. After centrifugation at 14,000 × g for 30 min to remove insoluble materials, cleared lysates were immunoprecipitated of FcγR by using human IgG, 32.2 and IV.3 mAb, and precipitated with test mAb as described previously (35). Bound materials were treated or not treated with N-glycanase (Genzyme), and samples were subjected to SDS-PAGE (45). For immunoblotting, immunoprecipitated proteins were separated by SDS-PAGE and transferred electrophoretically to a nitrocellulose Hybond-C (Amersham Pharmacia Biotech) filter for 18–20 h (46). The blots were incubated in blocking buffer composed of 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl (TBS) containing 3% bovine serum albumin and 0.1% Tween 20 and then incubated with anti-γ (1:500) for 2 h at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary Ab. Filters were developed using the Enhanced Chemiluminescence detection system (ECL, Amersham Pharmacia Biotech).

Common precipitation of Receptor-bound ^{125}I-Anti-FcR mAb—This was carried out as described in Ref. 47. Briefly, F(ab’)_2 fragments of A77 mAb or mouse IgG were labeled with Na^{125}I using the IODOGEN method (48). Cells (5 × 10^{9}) were incubated with ^{125}I-labeled test mAb (25–35 μg/ml) for 1 h, washed 3 times with PBS and 0.1% NaN_3, and then lysed in 0.5 ml of 1% digitonin buffer containing protease inhibitors as described above. After centrifugation, lysates were divided into two aliquots for 2-h incubations with either 20 μg of 4D8 anti-γ chain mAb or 50 μg of RAM IgA Ab coupled to Sepharose 4B. These amounts of antibodies had been identified as saturating concentrations for precipitation of labeled Ab complexes. The percentage of specifically precipitated counts was calculated for each Ab after subtraction of nonspecific counts obtained using either irrelevant IgG1-coupled beads or lysates that had been preincubated with a 100-fold excess of unlabeled anti-FcγR mAb (nonspecific counts were always <3%). For RBL transfectants, 3 μl of polyclonal rabbit anti-γ chain antiserum plus protein A-coupled beads were used to coprecipitate both rat and human γ chains (49). Normal rabbit serum was used to determine background precipitation.
Immunofluorescence and Flow Cytometry—RBL transfectants (1 × 10⁶) were stained with 10 μl of biotinylated A27 (Fab’₂) or irrelevant IgG1 Fab’₂ fragments (at 0.1 mg/ml) for 30 min at 4 °C followed by 10 μl of 1/50 diluted streptavidin PE (Southern Biotechnology Associates) as developing reagent. For IgA binding, cells preincubated with human IgG (10 mg/ml) to block FcγR were incubated with 10 μl of biotinylated purified IgA (0.5 mg/ml) for 1 h at 4 °C followed by streptavidin PE. For two-color immunofluorescence analysis, viable U937 cells (2 × 10⁶), preincubated with an excess of human IgG (10 mg/ml) to block FcγR, were stained directly with 10 μl of PE-labeled A59 anti-Fcγ mAb (0.1 mg/ml) or with an irrelevant PE-labeled IgG1 control for 30 min at 4 °C. After washing, cells were fixed with PBS containing 1% paraformaldehyde, permeabilized with PBS containing digitonin (10 μg/ml) for 5 min at 4 °C, and stained with anti-γ chain rabbit antisera (10 μl at 1:100 dilution) or a control rabbit serum for 30 min at 4 °C in PBS containing 0.05% Tween 20. After washes, cells were incubated with 10 μl of FITC-labeled goat anti-rabbit antibodies (25 μg/ml; human- and mouse-adsorbed, purchased from Southern Biotechnology Associates) for 30 min at 4 °C and analyzed by flow cytometry using a FACScanibur apparatus (Becton Dickinson). In some experiments, cytoplasmic molecules were evaluated on cells cytospun onto glass slides, fixed, and permeabilized for 20 min at −20 °C with 95% ethanol and 5% acetic acid solution, washed, and incubated for 20 min with 4D8, A59, or control IgG1 mAb (0.05 mg/ml). FITC-labeled anti-mouse Ig Ab (0.05 mg/ml) was added as a developing reagent and mounted on coverslips.

β-Hexosaminidase Assay—This was based on a method described previously (43). Briefly, transfectants were plated at 5 × 10⁴ cells in 100 μl of complete DMEM in the absence of G418 and sensitized with anti-dinitrophenyl IgE Abs (1/2000) or F(ab’₂) fragments of A77 mAb (0.01 mg/ml) for 4 h at 37 °C. Cells were washed in Hanks’ balanced saline solution containing 1% fetal calf serum and resuspended in the same buffer containing 100 μg/ml dinitrophenyl-human serum albumin (Sigma) or F(ab’₂) fragment RAM (40 μg/ml), respectively. To determine spontaneous release, cells were incubated in the absence of Ag (for FcR stimulation) or with irrelevant IgG1 Fab’₂ fragments (for FcR stimulation). Maximal release was determined with 100 nM PMA plus 1 μM ionomycin as stimulant. After incubation for 1.5 h, hexosaminidase

**Endocytic Pathways of IgA FcR with or without γ Chains**

**Fig. 2. Identification of γ-less FcγR on human myeloid cells.** 2 × 10⁶ blood neutrophils (panel A) and PMA-activated U937 cells (panel B) were surface labeled with Na125I, and the membrane proteins were solubilized using a 1% digitonin lysis buffer as described under “Experimental Procedures.” Lysates were divided into three aliquots and incubated with irrelevant IgG1 (lanes 1 and 4), anti-FcγR mAbs (lanes 2 and 5; A, A59 and B, A77 Fab’₂), or anti-γAbs (lanes 3 and 6; A, 4D8 and B, rabbit antiserum) plus RAM Ig Ab (panel A) or protein G (panel B) coupled to Sepharose 4B beads. Eight immunoadsorptions were performed with an excess of monoclonal (panel A) or polyclonal anti-γ chain (panel B) and followed by immunoprecipitations with test Abs. In panel A, immunoprecipitates were digested or not digested by N-glycanase, as indicated (N-gly) and analyzed by 10% SDS-PAGE (2ME+) and autoradiography. In panel B, immunoprecipitated 125I-surface proteins were separated by 12.5% SDS-PAGE (2ME−), transferred onto a nitrocellulose membrane, and analyzed by autoradiography (top) and immunoblotting (bottom) using anti-γ chain polyclonal Ab and horseradish peroxidase-conjugated anti-rabbit Ig Ab plus ECL.

**Fig. 3. A. γ-less FcγR represents the major fraction of FcγR molecules on the cell surface.** 5 × 10⁶ of U937 cells, monocytes, and neutrophils (empty, hatched, and filled bars, respectively) were incubated with 125I-labeled A77 anti-FcγR Fab’₂ fragments (25–35 μg/ml) for 1 h at 4 °C, washed, and solubilized in 1% digitonin lysis buffer. The lysates were divided into two aliquots and incubated for 2 h at 4 °C with either RAM Ig Ab or 4D8 mAb anti-γ chain coupled to Sepharose 4B. After washes, precipitated counts versus total counts were determined. Nonspecific precipitated counts were obtained using either irrelevant IgG1-coupled beads or lysates that had been preincubated with a 100-fold excess of unlabeled anti-FcγR. Bars (mean ± S.D. of at least three experiments performed in triplicate) show the calculated percentage of specifically precipitated 125I-labeled A77 bound to cell surface receptors. Panel B, time-dependent stability of FcγR-γ association in the mild detergent, digitonin. PMA-activated U937 cells were incubated with 125I-labeled A77 anti-FcγR Fab’₂ fragments for 1 h at 4 °C, washed, lysed (time 0) for 15 min on ice in 1% digitonin lysis buffer, and centrifuged for 15 min at 14,000 × g. Lysates were then incubated at 4 °C for different time periods and immunoprecipitated by 4D8 anti-γ chain mAb coupled to beads for 2 h at 4 °C and analyzed as described in panel A.
Internalization and Recycling Assays—This was performed as described elsewhere (50). Briefly, $10^5$ cells were incubated with $1 \mu g$ of $^{125}$I-F(ab')$_2$ fragments of A77 anti-FcR mAb for 1 h at 4 °C. After extensive washing, $10 \mu l$ of F(ab')$_2$ fragments of rabbit anti-mouse antibodies (1 mg/ml) was added for 30 min. Excess antibody was removed, and endocytosis was induced by incubating cells at 37 °C in RPMI 1640, 25 mM HEPES, 5% fetal calf serum for the times indicated. The reaction was stopped by placing the cells on ice. Any residual antibodies on the surface were removed by acid stripping (PBS, pH 2.5, at 4 °C for 5 min). This acid treatment routinely removes 85–90% of surface-bound anti-FcR F(ab')$_2$. After pelleting, cell-associated counts were detected in a gamma counter. In recycling experiments that cells were incubated with $1^{251}$I-polymeric IgA1 or $1^{251}$I-Fab fragments of A77 alone for 1 h on ice, washed, and then either treated or not treated for 20 min with 0.6 nM primaquine (Sigma) before incubation at 37 °C. Nonspecific counts were obtained by preincubating cells with a 100-fold excess of nonlabeled mAb or IgA. Data are expressed as percentages of total initial cell-associated counts and presented as the means ± S.D. of at least three separate experiments.

Measurement of IgA Proteolysis after Internalization—RBL-transfected cells were plated in the absence of G418 at 0.5 $\times 10^5$ cells/ml in 24-well Costar tissue culture plates. 24 h later, the cells were incubated with biotinylated, $^{125}$I-labeled dimeric IgA1x (1 $\mu g$/ml) in 0.1% bovine serum albumin, DMEM at 4 °C for 1 h. The medium was removed after 1 h, and the cells were washed three times at 4 °C. Cells were then incubated with streptavidin-conjugated PE (10 $\mu g$/ml) at 4 °C for 15 min to induce receptor aggregation. After washings, cells were cultured in DMEM containing 0.1% bovine serum albumin and 100 $\mu g$/ml unlabeled IgA1x for the times indicated. After incubation, the medium was removed, proteins were precipitated in 10% trichloroacetic acid, and acid-soluble and acid-insoluble radioactivities were counted in a gamma counter as described in Ref. 51.

Endocytosis Procedure by Confocal Microscopy—Adherent cells on glass slides were incubated at 4 °C for 30 min with 100 $\mu l$ of 0.1 mg/ml A77 anti-FcR F(ab')$_2$ fragments in PBS and 0.2% bovine serum albumin. After washings, cells were incubated with 100 $\mu l$ of 0.04 mg/ml RAM or FITC-coupled GAM for 30 min on ice. When indicated, cells carrying unlabeled antibodies were incubated further with 0.005 mg/ml GAR coupled to FITC to amplify aggregation. To visualize transferrin receptor-recycling vesicles, cells were cultured in serum-free DMEM for 30 min to deplete endogenous transferrin and incubated on ice with 100 $\mu g$/ml human transferrin coupled to Cy3 (kindly provided by Dr. A. Bement, CJF-97-10, Neckar Institute) together with anti-FcR mAb as above. The slides were either warmed to 37 °C for various times or kept at 4 °C. Cells were washed, fixed in 3% paraformaldehyde for 10 min, and quenched twice in PBS containing 1 mg/ml glycine. For intracellular $\gamma$ chain staining, cells were permeabilized with 0.05% saponin (Sigma) and stained with 3.5 $\mu g$/ml purified anti-$\gamma$ chain polyclonal Ab plus 3.5 $\mu g$/ml GAR coupled to Texas Red. To visualize the plasma membranes, cells were stained after endocytosis for 5 min at 4 °C with 100 $\mu g$/ml of wheat germ agglutinin coupled to Texas Red (38). After washing, the slides were mounted in 80% glycerol. Confocal double fluorescence acquisitions were made with a TCS4D confocal microscope based on a DM microscope interfaced with an argon/krypton laser. Simultaneous double fluorescence acquisitions were made with the 488 nm and 568 nm laser lines to excite FITC and Texas Red dyes using a 100 x oil-immersion Plan Apo objective (numerical aperture 1.4). The fluorescence was selected using appropriate double-fluorescence dichroic mirror and band-pass filters (52).

RESULTS

Identification of $\gamma$-less FcR on Human Myeloid Cells—Because FcR can be fully expressed at the surface of COS cells after transfection without the signaling $\gamma$ subunits (16), we investigated whether all FcR expressed on PMA-treated U937 cells were associated with the $\gamma$ chain homodimer by means of confocal microscopy. Because $\gamma$-less and $\gamma$-associated FcR could not initially be distinguished on the cell surface, we performed short term endocytosis of FcR-anti-FcR mAb complexes (FITC-labeled) to examine whether all internalized receptors colocalized with the $\gamma$ chain (Texas Red-labeled) in the vesicles. As shown in Fig. 1, two types of intracellular vesicles were detected in single cells, in which FcR was either colocalized (yellow) or not colocalized (green) with the $\gamma$ chain. These results strongly suggest the existence of $\gamma$-less and $\gamma$-associated
**Fig. 5.** FcR and γ chain are mostly expressed in the same cells. Viable U937 cells preincubated with an excess of human IgG to block FcR were stained directly with PE-labeled A59 anti-FcR mAb or with an irrelevant PE-labeled IgG1 control. After washes, digitonin-permeabilized cells were stained with anti-γ chain rabbit antiserum or a control rabbit serum and with FITC-labeled goat anti-rabbit antibodies as a developing reagent, as described under “Experimental Procedures.” Two-color immunofluorescence analysis was then carried out by flow cytometry. The values inside the boxes represent the percentage of cells.

**Table 1**

| Cell treatment                | Expression index | FcR + FcR-γ2 (total) | FcR-γ2 |
|------------------------------|------------------|----------------------|--------|
| IL-1β (50 units/ml)          | 1.05 ± 0.08      | 1.04 ± 0.10          |        |
| IFN-γ (50 units/ml)          | 1.17 ± 0.12      | 1.14 ± 0.18          |        |
| GM-CSF (100 pm)              | 2.10 ± 0.45      | 0.93 ± 0.02          |        |
| PMA (10−7 M)                 | 3.52 ± 0.38      | 1.82 ± 0.30          |        |
| Ionomycin (5 μM)             | 0.61 ± 0.06      | 1.04 ± 0.12          |        |

* Untreated or treated U937 cells (5 × 10⁶) were loaded with ¹²⁵I-labeled A77 anti-FcR mAb (25–35 μg/ml) for 1 h at 4 °C. After solubilization in 1% digitonin lysis buffer, counts specifically precipitated with 4D8 anti-γ chain mAb or anti-mouse IgG Ab were determined as described under “Experimental Procedures.” For total FcR, the expression index (mean ± S.E. of at least three experiments performed in triplicate) was calculated independently for each experiment by the ratio of cpm of treated cells to cpm of untreated cells. Total precipitated FcR binding sites in untreated cells was 6,102 ± 788 (determined from seven different experiments). For FcR-γ2 complexes, the expression index of the percentage of γ chain association with FcR was calculated independently for each experiment and expressed as the ratio of treated to untreated cells.

* Values indicate modulation of total FcR-γ2 complexes.

* Statistically significant differences (p < 0.05, Mann-Whitney test).

**To estimate the amounts of γ-less FcR we used a coimmunoprecipitation assay validated previously for FcRI (47).** Cells were loaded with ¹²⁵I-labeled anti-FcR mAb F(ab)2 fragments. Labeled receptor-Ab complexes were solubilized in the presence of 1% digitonin and precipitated with either anti-γ chain (4D8)- or anti-mouse Ig Ab. As shown in Fig. 3A, whereas total precipitable amounts of ¹²⁵I-Ab-FcR complexes using anti-mouse Ig Abs exceeded 70% (total FcR), those precipitated with the anti-FcR mAb were significantly lower on U937 cells, monocytes, and neutrophils (16 ± 5%; 21 ± 3%, and 29 ± 5%, respectively). The results were almost similar when ¹²⁵I-labeled Fab fragments of anti-FcR were used instead of F(ab)2 fragments in two comparative experiments (17 ± 0.5% versus 23.5 ± 1.5% in monocytes and 26 ± 3% versus 32 ± 1% in neutrophils, respectively). Similar results were also obtained using a rabbit anti-γ chain antiserum (data not shown). We then analyzed the time-dependent dissociation of the γ chain from the FcR in digitonin lysates. FcR-γ complexes were 55–75 kDa. Precipitation with an anti-γ chain antibody gave rise to similarly sized species. Treatment of anti-γ chain mAb-associated glycoproteins with N-glycanase resulted in 32- and 36-kDa bands that comigrated with those observed with the anti-FcR mAb (Fig. 2A). After extensive immunodepletion with anti-γ chain mAb, anti-FcR mAb-reactive molecules could still be precipitated (Fig. 2A). Similar results were obtained with PMA-treated U937 cells using a polyclonal anti-γ chain Ab (Fig. 2B), in which the same treatment eliminated most of the γ chain molecules (Fig. 2B, bottom). Conversely, immunoadsorptions with an anti-FcR mAb (A59) completely eliminated 55–75 kDa-4D8 mAb reactive proteins (not shown). These results reveal two forms of FcR, γ-less and γ-associated that are expressed on the surface of U937 cells and blood neutrophils. Because of the extensive immunoadsorption it was, however, impossible to quantify the two forms of FcR.

**To investigate whether the γ-less receptor population could also be detected in detergent extracts, we performed immuno-precipitation experiments using digitonin-solubilized cells because the FcR-γ2 interaction is resistant to digitonin treatment (26).** As shown in Fig. 2, immunoprecipitation of surface-iodinated FcR from blood neutrophils and PMA-treated U937 cells resulted in the appearance of the expected broad band of 55–75 kDa. Precipitation with an anti-γ chain antibody gave rise to similarly sized species. Treatment of anti-γ chain mAb-associated glycoproteins with N-glycanase resulted in 32- and 36-kDa bands that comigrated with those observed with the anti-FcR mAb (Fig. 2A). After extensive immunodepletion with anti-γ chain mAb, anti-FcR mAb-reactive molecules could still be precipitated (Fig. 2A). Similar results were obtained with PMA-treated U937 cells using a polyclonal anti-γ chain Ab (Fig. 2B), in which the same treatment eliminated most of the γ chain molecules (Fig. 2B, bottom). Conversely, immunoadsorptions with an anti-FcR mAb (A59) completely eliminated 55–75 kDa-4D8 mAb reactive proteins (not shown). These results reveal two forms of FcR, γ-less and γ-associated that are expressed on the surface of U937 cells and blood neutrophils. Because of the extensive immunoadsorption it was, however, impossible to quantify the two forms of FcR.

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very stable over a 24-h period, ruling out the possibility that partial association of FcαR with the γ chain was the result of their instability in detergents (Fig. 3B).

Partial Association of FcαR with the γ Chain Is Not Dependent on the Amounts of Expressed γ Chain—To examine the effect of the amounts of expressed γ chain, we transfected a previously established FcαR+ RBL transfectant (32) with the human γ chain. One clone (αγ) expressing large amounts of human γ chain was selected (Fig. 4, A and B). Coimmunoprecipitation experiments, using a polyclonal Ab that recognizes both rat and human γ chains, showed that in these transfectants the fraction of γ-associated receptors did not increase compared with cells transfected with FcαR only (Fig. 4C). As a control we coprecipitated the FceRI using the same anti-γ Ab. In agreement with previous observations (47), a major fraction of FceRI (>50%) was coprecipitated in these transfectants (data not shown). This demonstrated that the amount of anti-γ Ab used in the assay was not limiting. These results also indicated that association of the γ chain with FcαR did not depend on the amount of expressed γ chain.

FcαR and γ Chain Are Coexpressed in a Single Cell Population—The experiments shown in Fig. 1 suggested that both γ-less and γ-associated FcαR are expressed on the same cells. To further exclude the possibility that partial association was caused by heterogeneity in γ chain expression on a given cell population, we carried out two-color FACS analysis of cell surface FcαR and intracellular γ chain. Fig. 5 shows the expression of both FcαR and γ chain on U937 cells, before and after IFN-γ or PMA treatment. The majority of cells were FcαR+/γ−. Even though the expression level of both molecules was heterogeneous, the absence of two independent contour plots ruled out the existence of a subpopulation expressing only one of these proteins. The coexpression of FcαR and γ chain was also found in neutrophils as determined by cytoplasmic stainings (not shown).

Modulation of the γ Chain Association with FcαR on U937 Cells by Phorbol Esters and IFN-γ—Because a variety of agents have been described to modulate surface expression of FcαR (15, 17, 38, 53, 54), we investigated whether they also affected expression of FcαR-γ2 complexes as determined by coimmunoprecipitation. U937 cells were cultured with IL-1β, IFN-γ, GM-CSF, PMA, or ionomycin for 18 h. Table I shows that FcαR expression on the cell surface was enhanced significantly by PMA or GM-CSF, whereas ionomycin diminished receptor expression by about half. Interestingly, despite the lack of effect on FcαR surface expression, IFN-γ promoted a significant increase (about 1.5-fold) in γ chain association with FcαR. PMA also significantly favored γ chain association with FcαR (about 1.8-fold). Treatment with IL-1β, GM-CSF, or ionomycin had no significant effect on γ chain association with FcαR.

γ-less FcαR Binds IgA but Does Not Induce Exocytosis—We first established stable transfectants expressing either a wild-type or a mutant (R209L) FcαR by using the FcαR-negative rat mast cell line RBL-2H3. As shown in Fig. 6A, the selected transfectants expressed similar levels of FcαR. In contrast to the wild-type, the mutant receptor did not associate with en-
with PMA and Ca\(^{2+}\) 

### Endocytic Pathways of IgA FcR with or without \(\gamma\) Chains

**FIG. 7. Functions of \(\gamma\)-associated and \(\gamma\)-less FcR.** Panel A, release of \(\beta\)-hexosaminidase through FcR depends on their ability to associate with \(\gamma\) chains. RBL transfectants expressing either wild-type (open bars) or R209L (solid bars) FcR were incubated either with A77 anti-FcR mAb F(ab\(^\prime\))\(_2\) followed by RAM F(ab\(^\prime\))\(_2\) or mouse IgE plus diiutropheryl-human serum albumin, as described under “Experimental Procedures.” Secreted \(\beta\)-hexosaminidase was analyzed in the supernatants. Maximal release was obtained after incubation of cells with PMA plus ionomycin. Similar results were obtained with two other clones. Panel B, kinetics of FcR-mediated endocytosis are independent of their association with \(\gamma\) chains. RBL transfectants expressing either wild-type (closed circles) or R209L (open circles) FcR were loaded with \(^{125}\)I-A77 anti-FcR mAb F(ab\(^\prime\))\(_2\) at 4 °C for 1 h, washed, and incubated for 30 min with RAM F(ab\(^\prime\))\(_2\) fragments. Cells were warmed rapidly to 37 °C for the indicated time periods followed by acid treatment at 4 °C to remove cell surface-bound mAbs. Non-acid-releaseable counts were determined and expressed as percentage of total initial cell-associated counts and presented as the mean ± S.D. from at least three separate experiments.

Endogenous \(\gamma\) chains of RBL cells (Fig. 6B). Both types of receptor specifically bound monomeric and polymeric IgA1 or IgA2 molecules, as this binding was inhibited by My43 anti-FcR mAb (Table II). However, \(\gamma\)-less FcR bound more IgA than wild-type FcR, despite their similar levels of FcR expression (evaluated using mAb A77). The capacity of wild-type and mutant (R209L) receptors to mediate downstream events was examined by measuring the capacity of cells to degranulate in response to receptor stimulation. As a control the releasing capability of each individual transfectant was tested by stimulating cells through FcRI. Maximal release was obtained with PMA and Ca\(^{2+}\) ionophore. As shown in Fig. 7A, activation through mutant receptors did not lead to significant release of the granular enzyme, \(\beta\)-hexosaminidase, whereas the response to stimulation through wild-type FcR was comparable to that induced by FcRI.

**\(\gamma\)-less FcR-IgA Complexes Are Rapidly Endocytosed and Recycled to the Cell Surface**—We next examined endocytosis as a second function for both types of FcR. As shown in Fig. 7B, wild-type and mutant receptors internalized immune complexes at similar rates and amounts, indicating a potential endocytic function of \(\gamma\)-less FcR molecules. Analysis of endocytosis by means of confocal microscopy revealed numerous intracellular vesicles containing FcR in transfectants expressing \(\gamma\)-less or \(\gamma\)-associated FcR (Fig. 8). However, close inspection revealed a marked difference in the localization of intracellular endocytic vesicles between the mutant and wild-type FcR transfectants. The internalized mutant (R209L) FcR was localized very close to the periphery, whereas wild-type receptors were also found in vesicles deeper inside the cell. Colocalization experiments revealed that FcR was partially found within recycling vesicles that stained positively for transferrin receptors in both types of transfectants (Fig. 9). Recycling was also suggested by flow cytometry experiments in which high amounts of receptor complexes were still detectable on the cell surface even after endocytosis for 90 min (Fig. 10A). R209L-FcR mutant transfectants had significantly more anti-FcR mAb-receptor complexes on the cell surface than wild-type transfectants (exceeding 50% of initial fluorescence intensity values), suggesting a preferential role of \(\gamma\)-less receptors in recycling. To demonstrate receptor recycling, we took advantage of the recycling inhibitor primaquine. This drug blocks endocytic recycling vesicles from reaching the cell surface, thus accumulating the internalized ligand inside the cell, as described for recycling of internalized monomeric IgG by FcγRI (50). \(^{125}\)I-Polymeric IgA was bound to transfected FcR molecules and allowed to internalize for various periods in the presence or absence of primaquine. As shown in Fig. 10B, primaquine-induced accumulation of internalized \(^{125}\)I-polymeric IgA was significantly higher in R209L-FcR\(^{-}\) mutant transfectants than in cells expressing wild-type receptors, indicating that \(\gamma\)-less FcR recycles IgA toward the cell surface. **\(\gamma\)-less FcR Protects IgA from Degradation**—As it has recently been shown that \(\gamma\) chains mediate endocytic trafficking to lysosomes and are important for ligand degradation and antigen presentation (55), we examined the ability of wild-type and \(\gamma\)-less FcR to sort for IgA degradation. Biotinylated and iodinated dimeric IgA was bound to FcR on cells, followed by cross-linking using streptavidin PE to induce internalization. IgA proteolysis was monitored by determining the fraction of trichloroacetic acid-soluble radioactive counts in the supernatant between 30 and 120 min after endocytosis induction. As shown in Fig. 10C, no degradation of dimeric IgA was observed in R209L transfectants, whereas time-dependent IgA1 degradation was measured in wild-type FcR containing \(\gamma\)-associated receptors. No IgA degradation was detected in the absence of cross-linking in both types of transfectants (data not shown).

**DISCUSSION**

In this study, we report the existence of both \(\gamma\)-associated and \(\gamma\)-less surface FcR on blood monocytes and neutrophils as well as on U937 cells. This is demonstrated by three different technical approaches, which included confocal microscopy after FcR endocytosis, SDS-PAGE analysis of FcR immunodepleted in \(\gamma\) chains, and finally by coimmunoprecipitation assay of FcR using anti-\(\gamma\) Ab. Results of this last assay suggest that \(\gamma\)-less FcR represent a significant fraction of surface FcR molecules. The majority of cells express the two types of FcR. Although the positively charged arginine residue at position 209 of the FcR transmembrane domain is critical for the interaction with the \(\gamma\) chain (Ref. 28 and our results), the mechanism underlying and regulating the partial association of FcR with the \(\gamma\) chain is unknown. Genomic cloning has revealed a single gene encoding FcR (56), suggesting that the

\(^2\) R. C. Monteiro and U. Blank, unpublished results.
Fig. 8. Intracellular localization of internalized γ-associated and γ-less FcαR after 60-min endocytosis. Adherent RBL cells (wild-type (WT) and R209L mutant) were incubated successively for 30 min on ice with F(ab')2 of the anti-FcαR A77 mAb, with F(ab')2 fragments of RAM and with GAR coupled to FITC before incubation of the cells at 37 °C for 60 min as described under "Experimental Procedures." Cells were finally incubated or not with wheat germ agglutinin coupled to Texas Red (WGA) to delimit the plasma membrane. Cells were observed under a confocal microscope and optically sectioned at 1.5-μm intervals. A representative medial section of the horizontal slices is shown. No staining was observed when FITC-labeled secondary Ab or irrelevant IgG1 F(ab')2 was used.

Fig. 9. Colocalization of FcαR and transferrin receptors (TfR) in intracellular vesicles after 15-min endocytosis. Adherent RBL cells (wild-type (WT) and R209L mutant) were first cultured in serum-free DMEM for 30 min to deplete endogenous transferrin and then successively incubated with anti-FcαR A77 mAb, RAM, and GAR coupled to FITC on ice as described in Fig. 8. After FcαR staining, cells were incubated with 100 nm human transferrin coupled to Cy3 before incubation of the cells at 37 °C for 15 min. Cells were observed under a confocal microscope and optically sectioned at 1.5-μm intervals. A representative medial section of the horizontal slices is shown. No staining was observed when FITC-labeled secondary Ab or irrelevant IgG1 F(ab')2 was used.

Partial association of FcαR and the γ chain cannot be explained by the presence of a structurally different FcαR protein. This is further supported by experiments showing partial association in RBL cells transfected with the FcαR cDNA. It is also unlikely that intracellular amounts of γ chains are limiting for FcαR-γ2 expression because the amount of coprecipitated receptors with anti-γ was unchanged even when human γ chains were overexpressed. Rather, our data suggest that γ chain association with FcαR may be a regulated process susceptible to modulation by a variety of agents. In this context, it is interesting to note that IFN-γ favored γ chain association with FcαR independently of surface expression of the corresponding α chains, whereas phorbol esters increased both the amount of total FcαR and the percentage of FcαR-γ2. GM-CSF enhanced total FcαR but not FcαR-γ2. Furthermore, recruitment of the tyrosine kinases syk and Btk after FcαR activation is modulated by these agents (32) and may be a consequence of amounts of FcαR-γ2 complexes. Our results do not rule out the presence of another uncharacterized chain that would compete with the γ chain for association with the arginine residue in the FcαR transmembrane domain. Taken together these results indicate that the formation of multimeric FcαR is independent of the amounts of γ chains expressed and can be regulated by environmental factors that could be of physiologic relevance at inflammatory sites.

To examine the functional role of γ-less FcαR we established transfecants expressing γ-less FcαR (R209L mutants) or both types of receptor using the mast cell line RBL-2H3. We found that mutant γ-less FcαR bound IgA as efficiently, or even better, than wild-type receptors that contained FcαR-γ2. This seems to be different from FγRI and III where coexpression with γ chain enhances ligand affinity (57). Our results confirm that γ chain association with FcαR is not essential for IgA binding (16, 20). After cross-linking, cells expressing γ-less FcαR failed to release the granular marker β-hexosaminidase after FcαR aggregation, suggesting that γ chains play a key role in FcαR-mediated signaling pathways leading to exocytosis. A role for IgA in eosinophil degranulation has been demonstrated previously (58). Our study also corroborates previous observations on B cell transfecants expressing R209L FcαR in which downstream signals such as Ca2+ mobilization and IL-2 release were absent (28).

Although γ-less FcαR were unable to mediate downstream signaling, we found that both wild-type and mutant γ-less
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FcyR were able to endocytose after receptor clustering. Mutant γ-less FcyR was as efficient as wild-type receptors for internalization. Thus, endocytosis mediated by mutant γ-less FcyR does not depend on the presence of tyrosine-based motifs in the cytoplasmic tail. Indeed, this has also been shown for other FcR lacking the γ chain as is the case of FcγRI and FcγRIIb2 that mediate endocytosis of immune complexes (59–61). Our results point to major differences in endocytic pathways between these two forms of FcyR. In particular, internalized γ-less FcyR were only localized close to the periphery, whereas internalized wild-type FcyR (containing γ-less and γ-associated receptors) underwent deeper compartmentalization, suggesting γ chain sorting for the endo-lysosomal pathway. FcyR endo-lysosomal compartmentalization has been demonstrated previously on blood monocytes by their colocalization with cathepsin D (38). Furthermore, a role for γ chains in mediating endocytic sorting to lysosomes that leads to antigen presentation has been demonstrated recently for FcγR (55). Therefore, we focused on the characterization of biological functions mediated by γ-less FcyR. Intracellular vesicles containing γ-less FcyR colocalized with those containing transferrin receptors, suggesting that they were involved in recycling of FcyR and its bound ligand. Further evidence for the recycling of IgA by mutant γ-less FcyR was provided by the effects of primaquine, an inhibitor of receptor recycling. The significant increase in internalized polyclonal IgA by transfectants expressing mutant γ-less FcyR treated by primaquine is strongly indicative of FcyR-ligand recycling. Reflux of IgA toward the cell surface has been observed in blood monocytes from patients with alcoholic liver cirrhosis who have increased levels of serum IgA (38). Finally, our results indicate that mutant γ-less FcyR are unable to sort for IgA degradation even when cells are cultured for 2 h with cross-linked dimeric IgA, whereas cells expressing γ-associated FcyR degraded bound IgA.

Taken together, these findings point to the existence of myeloid cells expressing two types of FcyR with or without γ chains. They differ in the type of endocytic pathway used for the internalized ligand, which led us to propose the existence of an alternative mechanism that protects IgA from degradation. Because IgA bound to wild-type FcyR is not degraded without cross-linking, the use of each pathway may depend on the degree of aggregation of FcyR molecules. Cross-linking by IgA-immune complexes could thus increase numbers of FcyR-γ complexes delivering signals for downstream events such as degradation of IgA-immune complexes, processing, and antigen presentation. In agreement with this proposal, a previous study has shown that only large sized macromolecular IgA are efficiently and rapidly cleared from the circulation in humans, whereas clearance of smaller sized IgA polymers is considerably slower (62).

The protective role of γ-less FcyR may be important in view of maintaining serum IgA concentrations that would certainly counterbalance the rapid catabolism of IgA through other receptors such as the hepatocyte asialoglycoprotein receptor, which interacts with IgA through their carbohydrates (63, 64). Simultaneous expression of γ-associated and γ-less FcyR might thus increase cellular flexibility in carrying out alternative functions, independently, which either mediate IgA-antigen processing and presentation to major histocompatibility complex molecules or recycle the IgA monomer continuously to achieve serum IgA homeostasis.

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Fig. 10. γ-less FcyR recycles IgA and protects against degradation. Panel A, detection of FcyR-anti-FcyR mAb complexes on the cell surface after endocytosis. Wild-type (open circles) and R209L mutant (closed circles) FcyR transfectants were stained with 125I-polymeric IgA1 and analyzed by FACS. Results were calculated as follows 100 – [1100 × (x of A77 mAb incubated at 37 °C – x of negative control incubated at 37 °C)/x of A77 mAb incubated at 0 °C – x of negative control incubated at 0 °C] in which x is the computer mean fluorescence intensity value of each FACS profile. *p < 0.05 in Student’s t test. Panel B, increased intracellular accumulation of γ-less FcyR IgA complexes in the presence of primaquine, a recycling inhibitor. Wild-type (open bars) and R209L mutant (closed bars) FcyR transfectants were loaded with 125I-polymeric IgA1 at 4 °C for 1 h and then warmed rapidly to 37 °C in the presence or absence of 0.6 mM primaquine. Non-acid-releasable counts were determined at the time points indicated, calculated as a percentage of total initial cell-associated counts, and presented as the ratio of primaquine-treated to untreated cells. Results are expressed as the mean ± S.D. of three separate experiments. *p < 0.05 in Student’s t test. Panel C, degradation of IgA bound to FcyR. Cells expressing wild-type (open bars) or R209L mutant (closed bars) plated in triplicate for each time point were preincubated in serum-free medium for 30 min at 37 °C followed by incubation with 125I-labeled IgA for 1 h at 4 °C with streptavidin PE. Internalization of aggregated receptors was induced by incubating the cells at 37 °C. After the indicated times, trichloroacetic acid (TCA)-soluble radioactivity released into the medium was determined as described under “Experimental Procedures.” Each experimental point is expressed as a percentage of the total radioactivity recovered. Results of three experiments are presented as the mean ± S.D.
