

Filamin A Stabilizes Fc γ RI Surface Expression and Prevents Its Lysosomal Routing¹

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Filamin A, or actin-binding protein 280, is a ubiquitously expressed cytosolic protein that interacts with intracellular domains of multiple receptors to control their subcellular distribution, and signaling capacity. In this study, we document interaction between Fc γ RI, a high-affinity IgG receptor, and filamin A by yeast two-hybrid techniques and coimmunoprecipitation. Both proteins colocalized at the plasma membrane in monocytes, but dissociated upon Fc γ RI triggering. The filamin-deficient cell line M2 and a filamin-reconstituted M2 subclone (A7), were used to further study Fc γ RI-filamin interactions. Fc γ RI transfection in A7 cells with filamin resulted in high plasma membrane expression levels. In filamin-deficient M2 cells and in filamin RNA-interference studies, Fc γ RI surface expression was consistently reduced. Fc γ RI localized to LAMP-1-positive vesicles in the absence of filamin as shown by confocal microscopy indicative for lysosomal localization. Mouse IgG2a capture experiments suggested a transient membrane expression of Fc γ RI before being transported to the lysosomes. These data support a pivotal role for filamin in Fc γ RI surface expression via retention of Fc γ RI from a default lysosomal pathway. *The Journal of Immunology*, 2008, 180: 3938–3945.

Immune cells interact with Ab-Ag complexes through a variety of FcR (1). The class I IgG receptor (Fc γ RI) is constitutively expressed on monocytes, macrophages, and dendritic cells. Fc γ RI is a high-affinity receptor for IgG and exists as a multimeric complex comprised of a ligand-binding α -chain and the FcR γ -chain (2–4). Its *in vivo* role is illustrated by Fc γ RI^{-/-} mice that exhibit impaired Ab-dependent cellular processes such as bacterial clearance, phagocytosis, Ag presentation, and cytokine production (5, 6). For signaling, Fc γ RI relies both on the FcR γ -chain and the cytosolic domain of its α -chain (Fc γ RI-CY (cytoplasmic tail)⁴). Fc γ RI-CY facilitates MHC class II Ag presentation without active FcR γ -chain signaling (7), whereas deletion of Fc γ RI-CY retarded kinetics of endocytosis and phagocytosis, and abrogated Fc γ RI-triggered IL-6 secretion (8). Unlike the FcR γ -chain, Fc γ RI-CY does not contain ITAM or other tyrosine-containing signaling motifs.

Identification of interacting partners of Fc γ RI-CY may aid in deciphering signaling routes that control Fc γ RI function. We recently described an interaction between Fc γ RI-CY and periplakin that affects Fc γ RI-ligand binding, and downstream effector functions (9, 10). Previously, actin-binding protein 280, or filamin A

(filamin), was shown to coimmunoprecipitate with Fc γ RI (11). Filamin represents a homodimer composed of 280-kDa subunits that organizes actin filaments into orthogonal networks (reviewed in Refs. 12 and 13). In this study, we identified filamin in yeast two-hybrid screens using Fc γ RI-CY, and functionally characterized this interaction. Studies with a naturally filamin-deficient cell line (M2 cells), and a filamin-reconstituted subclone (A7 cells) indicated filamin to be crucial for cell morphology and locomotion, as well as subcellular localization and signaling of various receptors (14–21). To address the role of filamin for Fc γ RI biology, the subcellular distribution of Fc γ RI and filamin was studied in human monocytes. Stable Fc γ RI transfectants were generated in filamin-deficient M2 cells, and its filamin-reconstituted subclone A7, to assess the biological role of Fc γ RI-filamin interaction.

Materials and Methods

Constructs and chemicals

Fc γ RI (Gen Bank accession number L03418) was subcloned from pcDNA3 (22) (Invitrogen Life Technologies) containing neomycin resistance to pcDNA3.1 with zeocin resistance. The murine FcR γ -chain was *HindIII/XbaI* cloned into pCB7 containing hygromycin resistance (22). The TCR α -chain of pMX-TCR α -chain-internal ribosomal entry site-GFP (23), provided by Dr. S. B. Ebeling (Department of Hematology, University Medical Center, Utrecht, The Netherlands), was removed by *BamHI/NotI* digestion and replaced by Fc γ RI or Fc α RI subcloned from pCAV (24). PCR reagents for cloning were from PerkinElmer except for oligonucleotide primers (Isogen Bioscience). All constructs were verified by dideoxy sequencing using BigDye Terminators (Applied Biosystems) and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

Yeast two-hybrid screens

A MATCHMAKER human bone marrow cDNA library from BD Clontech was screened with Fc γ RI-CY as described in Ref. 22. Protein interactions were assessed by growth of transfected yeast cells on selective medium lacking leucine, tryptophane, and histidine, and a filter-lift β -galactosidase assay.

Cell culture, transfection, and bafilomycin A1 treatment

Human peripheral blood monocytes were isolated from healthy volunteers. Mononuclear cells were isolated from Ficoll gradients, and cultured with

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⁴ Abbreviations used in this paper: CY, cytoplasmic tail; siRNA, short interfering RNA; NP40, Nonidet P-40; ER, endoplasmic reticulum; EEA-1, early endosomal Ag-1; LAMP, lysosomal-associated membrane protein.

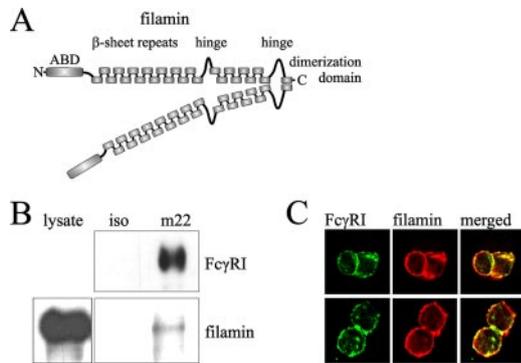


FIGURE 1. Fc γ RI interaction with filamin in yeast cells. *A*, Schematic representation of filamin; ABD, actin-binding domain. *B*, Coimmunoprecipitation of filamin and Fc γ RI. U937 cells were lysed after overnight stimulation with IFN- γ . Subsequently, immunoprecipitation was performed using an anti-Fc γ RI Ab (m22) or isotype control (iso). Western blotting was used to detect immunoprecipitated Fc γ RI (*top panel*) and coimmunoprecipitated filamin (*lower panel*). Experiments were performed three times, all yielding similar data. *C*, Subcellular localization of Fc γ RI and filamin in monocytes. Cells were stimulated overnight with 300 U/ml IFN- γ . Cells were adhered to glass slides, fixed in methanol, and Fc γ RI was stained with CD64 mAb H22 conjugated to FITC. Filamin was stained red. Green, red, and merged pictures of two stainings are shown. Colocalization is indicated in yellow.

IMDM containing L-glutamine (Invitrogen Life Technologies) supplemented with 10% FCS, penicillin, and streptomycin. After ~ 3 h at 37°C, nonadherent cells were removed, and adherent cells were incubated overnight with 300 IU/ml IFN- γ (IFN- γ 1b; Boehringer Ingelheim). Human melanoma cells selected for filamin deficiency (M2) and its filamin reconstituted subclone (A7) were cultured as described (14). Cells were transfected with fugene (Roche) according to the manufacturer's instructions. Stable Fc γ RI-transfected cells were selected with zeocin (500 μ g/ml). Inhibition of lysosomal maturation was accomplished by incubating cells with 100 nM bafilomycin A1 in medium for various time points. Carrier control represents DMSO 500-fold diluted in medium. U937 cells were cultured in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin (Invitrogen Life Technologies), and 100 μ g/ml streptomycin (Invitrogen Life Technologies).

Immunoprecipitation

For Fc γ RI immunoprecipitation, U937 cells were stimulated with IFN- γ overnight. Cells were then lysed in cold NP40 (NP40) buffer, 1% NP40 in PBS solution with Complete EDTA-free protein inhibitor mixture (Roche). Lysates were incubated overnight with either isotype control (Sigma-Aldrich) or m22 Abs (Medarex Europe) coupled to protein A/G beads (Santa Cruz Biotechnology). Subsequently, beads were washed in NP40 buffer, boiled in sample buffer, and subjected to Western blotting.

RNA interference

U937 cells were transfected with siGenome SMARTpool targeting human filamin A or with nontargeting siControl smartpool (Dharmacon) using Amaxa nucleofection. Cells were cultured in RPMI 1640 10% FCS and stimulated overnight with IFN- γ after 48 h. Seventy-two hours posttransfection, when filamin knockdown was maximal, the cells were stained with mAb anti-CD64 M22-FITC (Medarex) and analyzed on a FACSCalibur (BD Biosciences). Filamin protein levels were assessed by Western blotting using mAb1680 anti-human filamin A (Chemicon International) followed by goat anti-mouse IgG-HRP (Jackson ImmunoResearch Laboratories). For flow cytometric analysis of filamin protein levels, cells were fixed with 4% paraformaldehyde and stained in PBS containing 0.1% BSA, 0.1% saponin, 5% goat serum, and 5% rabbit serum using mAb 1680 and goat anti-mouse IgG-RPE (Southern Biotechnology Associates).

Capture experiments using mIgG2a-FITC

M2 or A7 stable transfectants were incubated with 2 μ g/ml mouse IgG2a-FITC (DakoCytomation) in RPMI 1640 10% FCS at 37°C or at 4°C. After 6 h, cells were washed with PBS and trypsinized. Subsequently, cells were split and washed three times with either normal RPMI 1640 2% FCS or

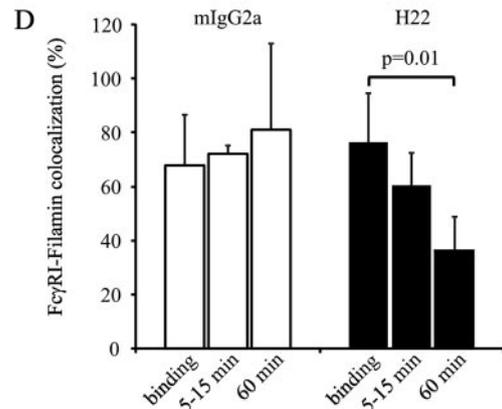
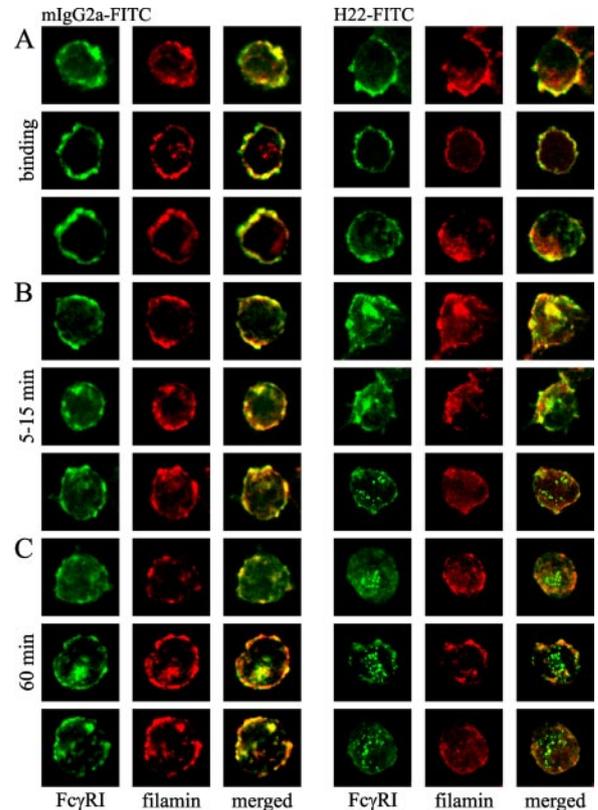


FIGURE 2. Subcellular localization of Fc γ RI and filamin in IFN- γ -stimulated monocytes. *A*, Monocytes were stimulated overnight with 300 U/ml IFN- γ . Cells were incubated with monomeric mIgG2a or mAb H22 (both FITC-conjugated) for 60 min at 4°C. Cells were then fixed, and stained for filamin. Three representative cells are shown. *B* and *C*, Fc γ RI ligands were added at 37°C at $t = 0$, and remained present throughout the experiment. Cells were fixed at different time points (*B*: 5–15 min, *C*: 60 min, three representative examples shown). Filamin was stained red. Experiments were repeated three times, all yielding similar data. *D*, The amount of Fc γ RI/filamin colocalization was quantified using Image J (see *Materials and Methods*), and the percentage of Fc γ RI/filamin colocalization was expressed as function of time ($n = 4$, Student's t test); 100% colocalization was determined by incubating H22-FITC or mIgG2A for 60 min at 4°C. Error bars indicate SD.

with RPMI 1640 2% FCS adjusted to pH 2.5 (acidic wash) (25). Cells were then analyzed using a FACSCalibur (BD Biosciences).

Immunofluorescence

Monocytes were isolated as described above, resuspended in PBS with 2 mM EDTA and 0.1% BSA, washed in medium, and adhered to poly-L-lysine-coated object glasses. For costainings with filamin, cells were fixed

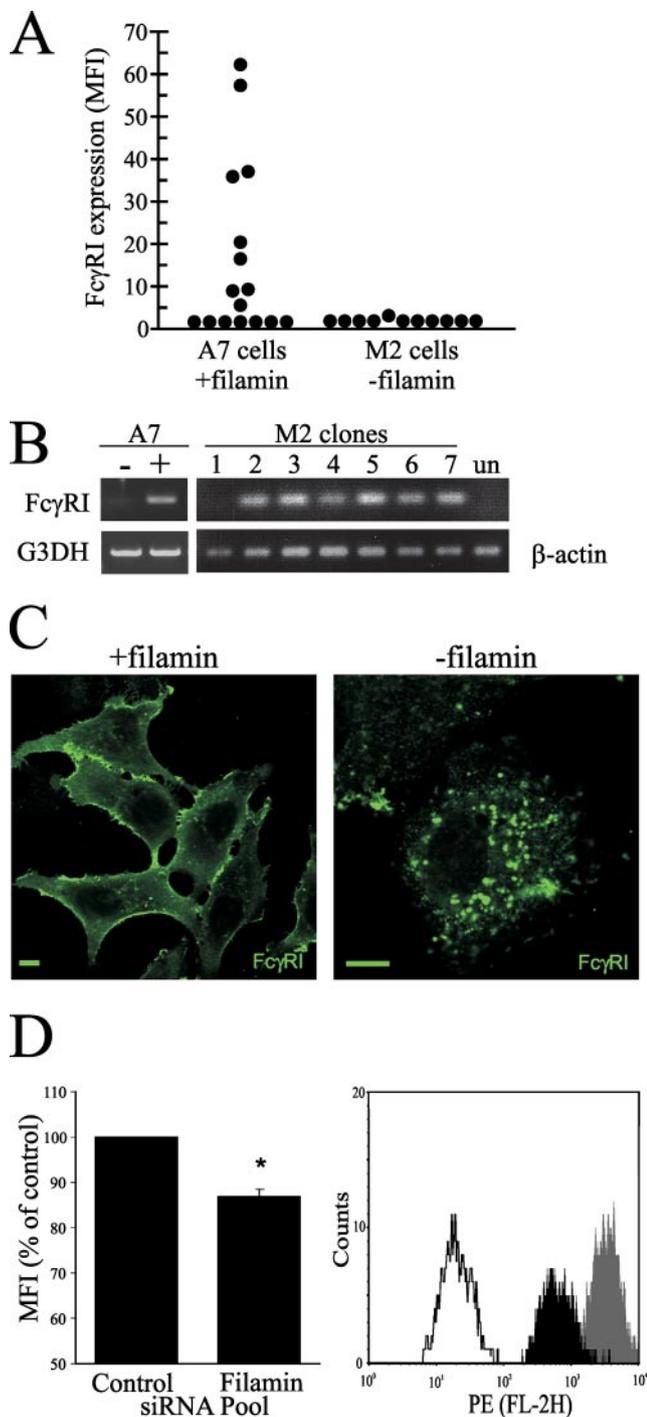


FIGURE 3. Fc γ RI surface expression on cells with or without filamin. **A**, Stable zeocin-resistant clones were randomly selected from three independent transfection experiments in cells with or without filamin. Fc γ RI expression was detected by CD64 mAb 10.1-FITC, and assessed by flow cytometry. Mean fluorescence intensities (MFI) are indicated. **B**, RNA was extracted from two A7 clones (left lane negative (-), and right lane positive (+) for Fc γ RI surface expression) and seven M2 clones, and RT-PCR for Fc γ RI was performed. **C**, Subcellular localization of Fc γ RI in filamin-deficient and filamin-reconstituted cells. Fc γ RI was stained by monoclonal 10.1-FITC after paraformaldehyde fixation (4) in filamin expressing clones (left panel) and filamin-deficient M2 cells (right panel). Bar marks 20 μ m. **D**, Relative Fc γ RI surface expression after filamin knockdown in U937 cells using siRNA (nontargeting control pool vs filamin targeting pool) was assessed by flow cytometry (left panel, *, $p = 0.004$, Mann-Whitney test, $n = 3$). Error bars indicate SD. Filamin knockdown was confirmed by Western blot (data not shown) and flow cytometry (right panel, one representative

in methanol at -20°C , washed extensively, and permeabilized in PBS containing 0.1% saponin, 0.2% BSA, 5% normal goat serum, and 5% normal rabbit serum. Fc γ RI was stained with 10 $\mu\text{g}/\text{ml}$ FITC-conjugated humanized anti-Fc γ RI mAb H22 (Medarex). Filamin was stained by mIgG1 anti-filamin (Chemicon International), and goat anti-mIgG1-Alexa 555 conjugates (Molecular Probes). For internalization experiments, adhered cells were incubated in medium with 10 $\mu\text{g}/\text{ml}$ FITC-conjugated H22 or mIgG2a for various time points at 37°C , fixed with methanol, and stained for filamin. Melanoma cells were grown on coverslips, fixed in PBS with 3% paraformaldehyde (or methanol when filamin was costained), and stained for Fc γ RI as described above, or with H22 F(ab')₂ followed by FITC-conjugated goat F(ab')₂ anti-human- κ -L chain (Southern Biotechnology Associates). Endoplasmic reticulum (ER) was indicated by rabbit anti-calreticulin, *cis*-Golgi by anti-GM130, and *trans*-Golgi by anti-p230; endosomal and lysosomal compartments were indicated with anti-early endosomal Ag-1 (EEA-1), anti-CD63, and anti-CD107a (all mIgG1 unless indicated otherwise; BD Biosciences). Secondary detection was with goat anti-mIgG1-Alexa 555 conjugates (Molecular Probes), or goat anti-rabbit CY3 conjugates (Jackson ImmunoResearch Laboratories). For transferrin internalization, cells were incubated with 40 $\mu\text{g}/\text{ml}$ transferrin conjugated to Alexa 555 (Molecular Probes) at 4°C , washed, and incubated at 37°C in medium for 15 min, fixed and processed for immunofluorescence as above. Slides were examined with a $\times 63$ planapo objective on a Leitz DMIRB fluorescence microscope (Leica) interfaced with a Leica TCS4D confocal laser microscope. Colocalization was quantified with Image J (<http://rsb.info.nih.gov/ij/>) using identical settings for each experiment (minimal pixel threshold 50, ratio 50%). Images from total cells were assessed for pixels that were positive both in the green (Fc γ RI) and red channel (subcellular markers, or filamin) and the total number of pixels in the green channel (Fc γ RI). The percentage of Fc γ RI colocalization with subcellular marker/filamin = colocalized pixels between Fc γ RI and a specific marker/total Fc γ RI pixels $\times 100\%$.

Flow cytometry

M2 and A7 cells were detached 48 h posttransfection, and stained in PBS containing 0.1% BSA, 2 mM EDTA, and 10% normal mouse serum. Cells were washed, and surface Fc γ RI, or total Fc γ RI was detected with mAb 10.1-FITC (Serotec) in the absence or presence of 0.1% saponin, respectively, at 4°C . Cells were washed and analyzed with a FACSCalibur (BD Biosciences). Surface expression was scored positive when it exceeded three times background staining of untransfected cells. When Fc γ RI expression was compared with Fc α RI expression, Fc γ RI and Fc α RI were stained in 40 μl with 20 $\mu\text{g}/\text{ml}$ mIgG1 clone 10.1 and A59, respectively, followed by 10 $\mu\text{g}/\text{ml}$ goat anti-mouse PE conjugated (Jackson ImmunoResearch Laboratories).

RT-PCR

RNA was extracted from cells with Qiagen RNeasy midi columns (Qiagen), and reverse transcribed with oligo-dT primers of a GeneAmp RNA PCR kit (Applied Biosystems). Fc γ RI was amplified by 33 cycles as described in Ref. 22. Actin was amplified by 25 cycles with 5'-gtggggcgc cccagcaccag-3' and 5'-ctccttaatgtcacgcacgatttc-3' under standard conditions for PCR.

Western blot

A total of 1×10^5 cells were lysed in reducing Laemmli sample buffer, and proteins were separated with SDS-PAGE (12% gel). Proteins were transferred to nylon membranes, and stained for Fc γ chain (Upstate Biotechnology) that was detected by goat anti-rabbit conjugated to HRP (Pierce). ECL and Biomax films were obtained from Amersham Biosciences.

Results

Filamin interacts directly with cytoplasmic domains of multiple receptors, and can profoundly affect their function (13). We found filamin (a schematic representation of filamin is shown in Fig. 1A) to interact with Fc γ RI using yeast two-hybrid screens on a bone marrow cDNA library. Cotransfection of the filamin-containing cDNA with empty bait plasmids did not allow yeast cells to grow

(experiment). Open histogram depicts background of secondary Ab; gray histogram depicts filamin staining in cells transfected with control pool; and black histogram shows cells transfected with filamin targeting pool.

on selective medium (data not shown). Importantly, we confirmed this interaction by coimmunoprecipitating filamin via Fc γ RI from IFN- γ -stimulated U937 cells expressing both proteins endogenously (Fig. 1*B*). We next analyzed the subcellular localization of Fc γ RI and filamin in primary IFN- γ -stimulated monocytes (Fig. 1*C*). A significant portion of Fc γ RI and filamin colocalized at the plasma membrane when cells were fixed and stained with the CD64 mAb H22.

When monocytes were incubated with monomeric ligand (mIgG2a-FITC), or cross-linking Fc γ RI mAb (H22-FITC) that can trigger Fc γ RI internalization (26, 27), extensive colocalization was observed at the plasma membrane, and on mIgG2a-positive intracellular compartments (Fig. 2, *A–C*, left panels). Some filamin still colocalizes intracellularly with Fc γ RI at early time points (5–15 min) after H22-FITC incubation (Fig. 2, *A* and *B*, right panels). However, H22-FITC induced drastic Fc γ RI-filamin dissociation at later time points (Fig. 2, *A–C*, right panels, and *D*). Together, these results suggest that Fc γ RI and filamin predominantly interact at the plasma membrane, and to a minor extent at an intracellular compartment.

Next, we studied Fc γ RI in a filamin-deficient cell system, and stably transfected filamin-deficient M2 cells, and its filamin-reconstituted subclone A7 with the α -chain of Fc γ RI (Fc γ RI) (14). From three independent Fc γ RI transfections, 16 zeocin-resistant A7 clones and 12 M2 clones were selected. Fc γ RI surface expression in A7 cells was observed in ~50% of subclones (8 of 16 clones; Fig. 3*A*). Surprisingly, Fc γ RI cell surface expression in 12 subcloned M2 cells was extremely low or even undetectable, albeit six of seven M2 transfectants expressed Fc γ RI at the transcript level (Fig. 3*B*). This indicated filamin to be an important determinant of Fc γ RI surface expression (percentage Fc γ RI-positive A7 clones were compared with M2 clones, Fisher's exact test $p = 0.0083$). Next, we assessed Fc γ RI expression at the subcellular level in A7 and M2 cells by confocal microscopy (Fig. 3*C*). In the presence of filamin, Fc γ RI surface expression was evident, although some intracellular staining could be detected. In M2 cells lacking filamin, Fc γ RI-specific staining predominantly localized intracellularly, and was more difficult to detect suggesting lower total Fc γ RI protein levels (Fig. 3*C* (and see Fig. 7*A*)). M2 cells were confirmed to be filamin negative by Western blot and immunofluorescence analyses (data not shown). Fc γ RI colocalized with filamin at the plasma membrane, and to some extent at intracellular vesicles in A7 cells (data not shown; $n = 3$). Knockdown of filamin using short interfering RNA (siRNA) in U937 cells lead to a decreased cell surface expression of endogenous expressed Fc γ RI (Fig. 3*D*, left panel; Mann-Whitney U test, $p = 0.004$). Filamin knockdown was confirmed by flow cytometry (Fig. 3*D*, right panel) and Western blot (data not shown).

To assess whether the defective plasma membrane expression in M2 cells was selective for Fc γ RI, we included the receptor for IgA—FcaRI or CD89—as a control in transient transfections of M2 cells and A7 cells with Fc γ RI. Both receptors were expressed from vectors that coexpressed enhanced GFP. Surface expression levels of Fc γ RI, and FcaRI were comparable upon transfection in cells with filamin (Fig. 4*A*, $n = 3$). Transfection efficiencies were comparable as indicated by GFP signals. Cells without filamin, however, displayed impaired Fc γ RI surface levels, whereas FcaRI surface levels were unaffected. Control staining was performed on mock GFP-transfected cells with isotype controls (Fig. 4*A*) and untransfected cells (data not shown, $n = 3$). Similar differences were apparent at days 3 and 4 posttransfection (data not shown, $n = 2$).

In mice, *in vivo* surface expression of many FcR, including Fc γ RI, has been found to (partly) rely on the associated FcR

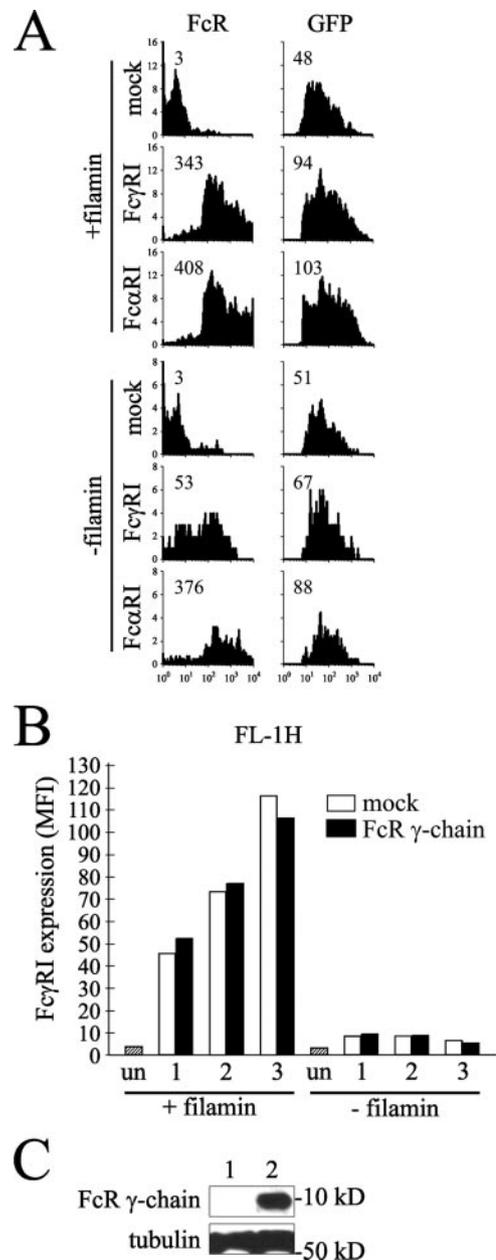


FIGURE 4. *A*, Transient transfection of M2 and A7 cells with Fc γ RI and FcaRI cDNA. Cells were transfected with Fc γ RI and FcaRI expressed from plasmids that coexpressed enhanced GFP. Fc γ RI and FcaRI were stained with mAb M22 and A59, respectively, followed by incubation with PE-labeled anti-mouse IgG and flow cytometry. Gates for viable GFP-positive cells were applied, and cells were assessed for FcR and GFP expression 48 h posttransfection. Histograms of GFP-gated cells stained for Fc γ RI and FcaRI are shown in the left column, associated GFP levels are shown on the right. Cells were transfected with the original vector that coexpressed TCR α -chain and GFP to check for staining specificity. The mock cells shown in this figure were transfected with the original vector that coexpressed TCR α -chain and GFP and stained with Abs against Fc γ RI. However, essentially the same results were obtained when these cells were stained with Abs against FcaRI (data not shown). MFI are shown in the top left corner of each histogram. Three experiments were performed, yielding essentially identical results. *B*, Effect of FcR γ -chain transfection on Fc γ RI surface expression. Three filamin-positive and filamin-negative lines that expressed Fc γ RI transcripts were transfected with FcR γ -chain (■) or mock plasmid (□). ▨, Untransfected parental cells (un). Cells were stained for surface Fc γ RI by mAb 10.1-FITC and assessed by flow cytometry (MFI are indicated). Two experiments yielded similar data. *C*, Western blot for FcR γ -chain, and tubulin after transfection of mock (lane 1) or FcR γ -chain (lane 2) plasmids in M2 cells. Transfected A7 cells showed similar levels of FcR γ -chain. Tubulin was used as loading control.

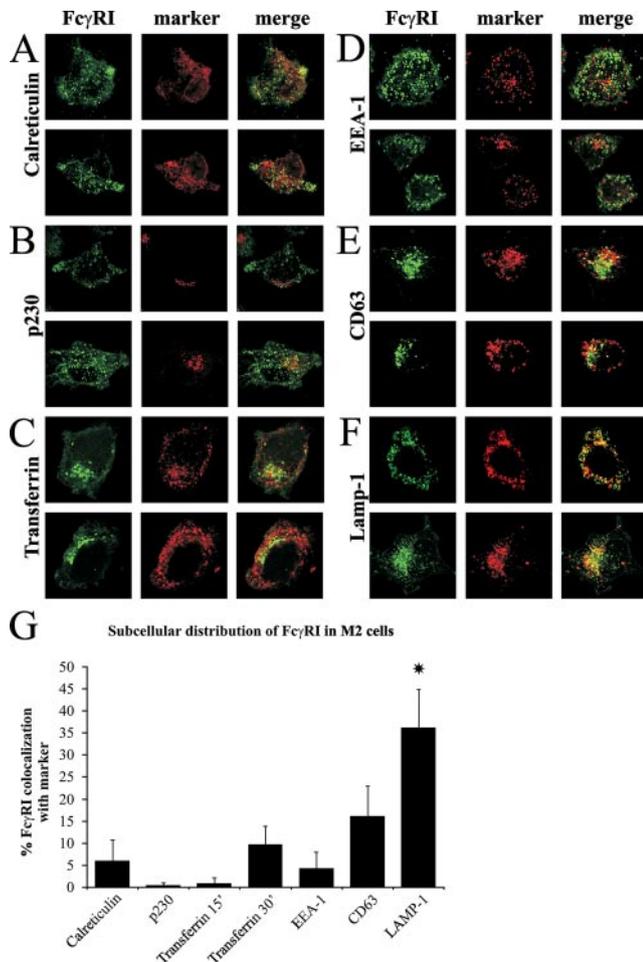


FIGURE 5. Intracellular location of Fc γ RI in cells without filamin. M2 cells were stained for markers involved in plasma membrane afferent pathways, and the endosomal/lysosomal pathway. Fc γ RI was stained in green (H22-FITC), all other markers in red. Panels were stained as follows: *A* was stained for calreticulin, *B* for p230 (Golgi), *C* for transferrin uptake, *D* for EEA-1, *E* for CD63, *F* for LAMP-1/CD107a. *G*, Percentage colocalization with subcellular markers was calculated using Image J as described in *Materials and Methods*. Cells from three independent experiments were quantified. *, Statistical significance between LAMP-1 and other markers (Student's *t* test, $p < 0.05$).

γ -chain (3, 4, 28). Therefore, to address whether the FcR γ -chain was capable of rescuing the impaired surface expression in this system, it was transiently cotransfected in three Fc γ RI-transfected M2- and A7-subcloned cell lines. No appreciable differences were observed for surface Fc γ RI upon mock or FcR γ -chain transfection (Fig. 4*B*, $n = 2$). FcR γ -chain transfections were confirmed by Western blot, and one representative sample is shown in Fig. 4*C*.

Next, we set out to identify the intracellular compartment in which Fc γ RI resides in filamin-deficient cells by immunofluorescence and electron microscopy (Fig. 5). We tested a panel of Abs recognizing compartments that are involved in afferent plasma membrane pathways such as calreticulin (Fig. 5*A*) for the ER, and p230 (Fig. 5*B*) and GM-130 (data not shown) for Golgi. Endosomal compartments were analyzed by transferrin uptake (Fig. 5*C*), and mAb stainings for EEA-1 (Fig. 5*D*), lysosomal integral membrane protein-1 (CD63; Fig. 5*E*), and lysosomal-associated membrane protein (LAMP-1)/CD107a (Fig. 5*F*). We did not observe a clearly defined compartment in which Fc γ RI accumulated using these markers, suggestive for a transient passage through the com-

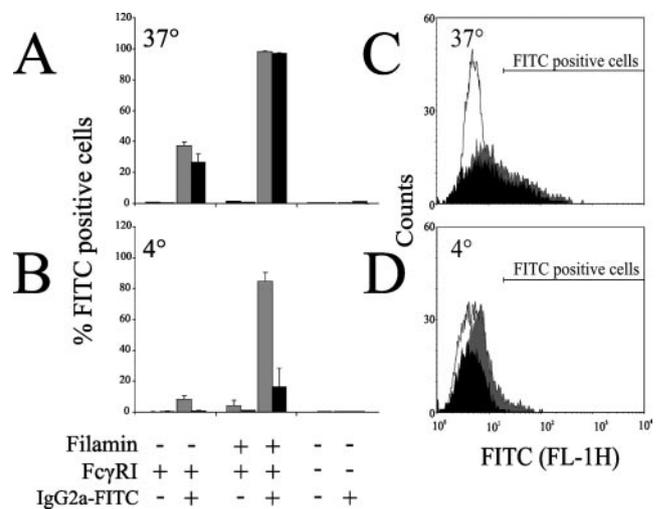


FIGURE 6. Mouse IgG2a uptake by M2 and A7 cells expressing Fc γ RI. Stable clones of M2 (M2 Fc γ RI), A7 (A7 Fc γ RI), and parental M2 cells (M2) were incubated with or without mouse IgG2a-FITC for 6 h. After incubation at 37°C (*A*) or at 4°C (*B*), cells were washed at either normal pH (□) or at pH 2.5 (■) to remove surface-bound IgG2a. Cells were analyzed by flow cytometry. Bar graphs show the percentage of FITC-positive cells defined by regions drawn in *C* and *D*. Bars represent the mean of three experiments. Error bars indicate SD. *C* and *D*, Histograms from one representative experiment of M2 cells expressing Fc γ RI incubated with mouse IgG2a-FITC at 37°C (*C*) or at 4°C (*D*). Open histograms represent cells without mIgG2a; gray histograms depict cells incubated with mIgG2a and washed at normal pH; black histograms represent cells washed at pH 2.5.

partments tested. Most colocalization between Fc γ RI and a subcellular marker was observed for the lysosomal marker LAMP-1 as indicated by quantification of colocalized signals of cells from three different experiments (Fig. 5*G*). Treatment of cells with nocodazole, which disrupts microtubuli and disperses cellular organelles, reduced some colocalization of Fc γ RI and LAMP-1 (data not shown, $n = 2$). In A7 cells, intracellular Fc γ RI could be found in similar compartments as for cells without filamin, although A7 cells displayed plasma membrane accumulation (data not shown).

To test whether in the absence of filamin, Fc γ RI is indeed transported to the plasma membrane before entry into a lysosomal pathway, we performed mIgG2a-FITC capture experiments. M2 and A7 transfectants were incubated with mIgG2a-FITC at 37°C (Fig. 6, *A* and *C*) to allow binding and endocytosis or at 4°C (Fig. 6, *B* and *D*) to allow only binding to Fc γ RI. M2 and A7 transfectants became FITC positive after incubation with mIgG2a-FITC. Washing cells with low pH removed surface-bound IgG (Fig. 6, *B* and *C*) but only partly abrogated the FITC signal when cells were incubated at 37°C, suggesting endocytosed mIgG2a-FITC in both M2 (Fig. 6, *A* and *C*) and A7 (Fig. 6*A*) transfectants. Uptake of mIgG-FITC appeared dependent on Fc γ RI as the untransfected parental M2 cells did not obtain FITC signal. In combination with the colocalization experiments, these data suggested filamin to stabilize Fc γ RI at the plasma membrane and to prevent entry of Fc γ RI into a lysosomal pathway.

Total Fc γ RI protein levels in M2 and A7 clones were assessed by flow cytometry upon cell permeabilization with saponin (Fig. 7*A*). Fc γ RI protein levels were significantly reduced in the absence of filamin. To demonstrate that these cells produced significant levels of Fc γ RI, and that the low Fc γ RI amounts were a consequence of lysosomal degradation, we incubated cells with bafilomycin A1. Bafilomycin A1 inhibits the vacuolar-type H⁺-ATPase

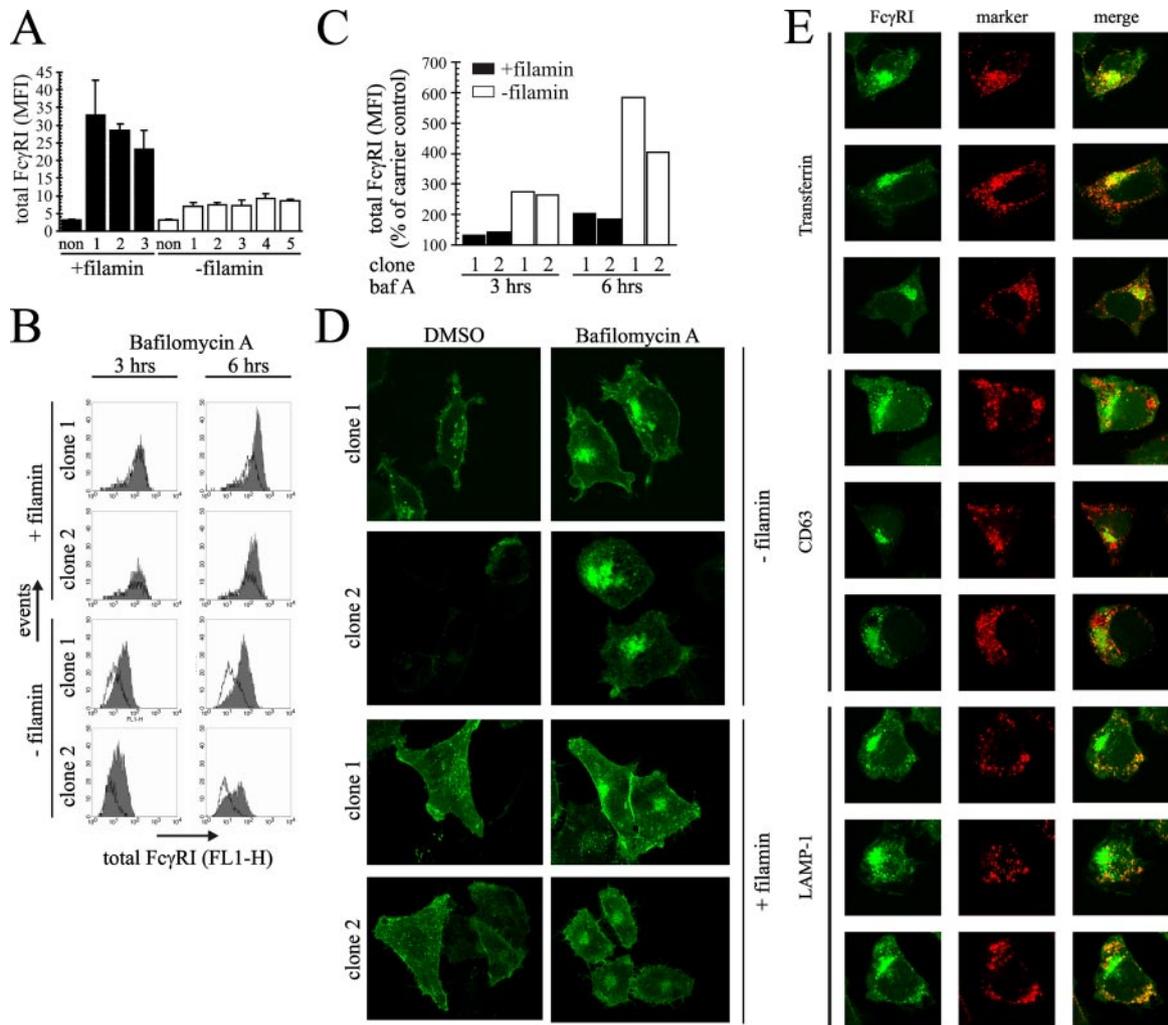


FIGURE 7. Filamin prevents lysosomal degradation of Fc γ RI. *A*, Filamin-positive and -negative lines that expressed Fc γ RI transcripts were stained for total Fc γ RI in the presence of 0.1% saponin. ■, A7 clones; □, M2 clones; un, untransfected cells. Numbers represent independent clones of Fc γ RI transfectants. Average mean values of four independent experiments are shown, error bars indicate SE, of the mean. *B*, Two Fc γ RI-expressing clones with or without filamin were incubated with bafilomycin A1 (100 nM) for 3 or 6 h. Total Fc γ RI was assessed as in *A*. Black-lined open histograms represent total Fc γ RI after incubation in carrier control (DMSO 500-fold diluted); gray-filled histograms represent total Fc γ RI after bafilomycin A1 treatment. Representative data of three independent experiments are shown. *C*, Relative increase of total Fc γ RI after incubation of cells with bafilomycin A1. Two Fc γ RI-transfected cell lines with (■) or without (□) filamin were compared. For each cell line, total Fc γ RI levels after DMSO incubation was set at 100%. *D*, Fc γ RI-transfected M2 and A7 cells were assessed by confocal microscopy after 2 h incubation with bafilomycin A1 or DMSO. Fc γ RI was stained by mAb 10.1 FITC-conjugated. *E*, Intracellular location of Fc γ RI in cells without filamin after bafilomycin A1 treatment. M2 cells were stained for markers involved in endosomal (transferrin uptake) and lysosomal (CD63, LAMP-1) pathways. Fc γ RI was stained in green (H22-FITC), all other markers in red.

that regulates endosomal pH, and hence prevents intracellular degradation of endocytosed cargo and receptors (35, 37). Fc γ RI levels in cells without filamin increased profoundly upon incubation with bafilomycin A1 (Fig. 7, *B* and *C*, $n = 3$), but not with the proteasomal inhibitor lactacystin (data not shown). After 6 h, Fc γ RI levels almost reached those of A7 cells incubated with carrier control. Some accumulation of Fc γ RI after 6 h was observed for Fc γ RI in A7 cells, suggesting limited lysosomal Fc γ RI degradation in the presence of filamin. No changes were observed for Fc γ RI surface levels in the presence of bafilomycin A1, suggesting Fc γ RI internalization not to be affected (data not shown, refs 35,37). Bafilomycin A1 clearly elevated intracellular staining of Fc γ RI in cells without filamin (Fig. 7*D*). Upon bafilomycin A1 treatment A7 cells showed an increase of intracellular staining of Fc γ RI as well (Fig. 7*D*) which coincided with flow cytometric analysis of total protein levels (Fig. 7*C*). Interestingly, bafilomycin A1 treatment caused Fc γ RI to accumulate predominantly in the

endosomal compartment (Fig. 7*E*, transferrin staining), although some colocalization was also seen with lysosomal markers CD63 and LAMP-1.

Discussion

To identify protein effectors of Fc γ RI, we performed yeast two-hybrid screens and identified filamin, an actin-binding protein. This interaction was confirmed in coimmunoprecipitation experiments with endogenously expressed Fc γ RI and filamin (Fig. 1*B*). We found filamin to partially colocalize with Fc γ RI in primary monocytes after fixation (staining with mAb recognizing Fc γ RI, independently of bound ligand), or by addition of monomeric IgG, and an Fc γ RI-cross-linking mAb at 37°C before fixation (Figs. 1*C* and 2). Most colocalization was observed at the plasma membrane where filamin may act to stabilize Fc γ RI surface expression by tethering Fc γ RI to actin, and preventing Fc γ RI to internalize, as was suggested by our studies in filamin-deficient

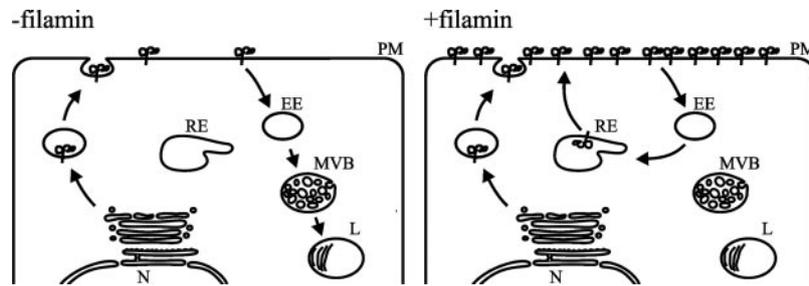


FIGURE 8. Model of Fc γ RI-filamin interactions. In the absence of filamin (*left panel*), Fc γ RI is capable of reaching the plasma membrane after synthesis, but is internalized rapidly, and routed by default toward lysosomes where it becomes degraded. In the presence of filamin (*right panel*), Fc γ RI is routed toward the plasma membrane after synthesis in the ER. Filamin interactions with Fc γ RI stabilize plasma membrane localization of Fc γ RI by reducing internalization of ligand-free Fc γ RI. Internalized Fc γ RI may be sequestered into a recycling pathway by filamin to further support plasma membrane expression, and prevent unwanted lysosomal degradation of receptor and cargo; EE, early endosome; L, lysosome; MVB, multivesicular body; N, nucleus; PM, plasma membrane; RE, recycling endosome.

cells. As monomeric IgG induces Fc γ RI internalization into a recycling route that prevents its lysosomal degradation (29), intracellular Fc γ RI-filamin interactions upon monomeric IgG incubation may contribute to its surface expression by retaining Fc γ RI in a recycling pathway, similar as described for calcitonin receptor-filamin interactions (19). Although our data suggest that IgG-occupied Fc γ RI can interact with filamin (Fig. 2), a previous report indicated filamin to preferentially interact with ligand-free Fc γ RI (11). As suggested by these authors, effective IgG-induced dissociation of Fc γ RI-filamin might require larger IgG complexes that may coincide with induction of phagocytosis (11).

We observed Fc γ RI surface expression *in vitro* to depend on filamin, shown in a transfection model of cells that differed by filamin expression and by RNA interference (Figs. 3–5). Routing of Fc γ RI toward the plasma membrane in filamin-negative cells appeared normal: Fc γ RI did not accumulate in compartments that are involved in afferent plasma membrane transport such as ER and Golgi, its expression was insensitive to coexpressed FcR γ -chain, and some surface expression was observed in transient transfection experiments. Moreover, capture experiments suggested transitory Fc γ RI surface expression (Fig. 6) in the absence of filamin. However, a significant proportion of Fc γ RI localization was confined to (pre)lysosomal compartments in M2 cells as apparent from confocal studies (in the absence of cross-linking ligand; Fig. 5). Fc γ RI protein levels were also highly sensitive to bafilomycin A1 in cells without filamin (Fig. 6). Interestingly, bafilomycin A1 treatment of M2 cells caused Fc γ RI to accumulate predominantly in the endosomal compartment (Fig. 7E). This coincides with previous publications showing that acidification is critical for fusion of yeast vacuoles (30, 31). Capture experiments and colocalization studies suggested that Fc γ RI does not accumulate on the cell surface, but transiently passes through this compartment to be finally degraded in lysosomal structures. Fc γ RI is unique among multisubunit FcR, and harbors intracellular residues that facilitate MHC class II presentation after immune complex triggering (7). This pathway may be inhibited by filamin activity in resting immune cells to facilitate Fc γ RI surface expression, and prevent undesired degradation and presentation of Ags (a model is presented in Fig. 8).

Previous studies have demonstrated *in vitro* surface expression of Fc γ RI, in contrast to Fc γ RIII and Fc ϵ RI, to be independent of coexpressed FcR γ -chain (32, 33). Fc γ RI lacks ER retention motifs present in the α -chains of Fc γ RIII and Fc ϵ RI that are masked by the FcR γ -chain to allow surface expression (33–35). This was supported by the inability of the FcR γ -chain to rescue Fc γ RI surface expression in our experiments (Fig. 4). *In vivo*, surface

expression of Fc γ RI, Fc γ RIII, and Fc ϵ RI is reduced in FcR γ -chain-deficient mice, albeit Fc γ RI is detectable at ~20% of wild-type levels (3–5). This may indicate that in these cells the remaining Fc γ RI is stabilized by filamin, or that coexpressed FcR γ -chain modulates filamin activity *in vivo*.

We recently reported periplakin to interact with the membrane-proximal domain of Fc γ RI under similar conditions as described here for filamin, albeit periplakin and Fc γ RI did not colocalize on intracellular vesicles (10, 22). Although it remains unclear how periplakin and filamin interact in Fc γ RI functioning, the present data may suggest these proteins to affect separate Fc γ RI functions. Blockade of Fc γ RI-periplakin interaction by overexpressed C-terminal periplakin or blocking peptides modulated Fc γ RI ligand-binding capacity and downstream effector functions but not surface expression (10, 22). Both proteins can also act as a cytoskeletal-associated scaffold for signal transducers (12, 36, 37), suggesting that periplakin and filamin may coordinate different signaling pathways. The data presented here point at a vital role for filamin in Fc γ RI biology by stabilizing surface expression, and retention of Fc γ RI from a default lysosomal pathway that mediates Fc γ RI degradation.

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Disclosures

The authors have no financial conflict of interest.

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