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*J Immunol* 2006; 176:3603-3610; ;  
doi: 10.4049/jimmunol.176.6.3603  
<http://www.jimmunol.org/content/176/6/3603>

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Signaling through Mutants of the IgA Receptor CD89 and Consequences for Fc Receptor $\gamma$ -Chain Interaction<sup>1</sup>

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The prototypic receptor for IgA (Fc $\alpha$ RI, CD89) is expressed on myeloid cells and can trigger phagocytosis, tumor cell lysis, and release of inflammatory mediators. The functions of Fc $\alpha$ RI and activating receptors for IgG (Fc $\gamma$ RI and Fc $\gamma$ RIII) are dependent on the FcR  $\gamma$ -chain dimer. This study increases our understanding of the molecular basis of the Fc $\alpha$ RI-FcR  $\gamma$ -chain transmembrane interaction, which is distinct from that of other activatory FcRs. Fc $\alpha$ RI is unique in its interaction with the common FcR  $\gamma$ -chain, because it is based on a positively charged residue at position 209, which associates with a negatively charged amino acid of FcR  $\gamma$ -chain. We explored the importance of the position of this positive charge within human Fc $\alpha$ RI for FcR  $\gamma$ -chain association and Fc $\alpha$ RI functioning with the use of site-directed mutagenesis. In an Fc $\alpha$ RI R209L/A213H mutant, which represents a vertical relocation of the positive charge, proximal and distal FcR  $\gamma$ -chain-dependent functions, such as calcium flux, MAPK phosphorylation, and IL-2 release, were similar to those of wild-type Fc $\alpha$ RI. A lateral transfer of the positive charge, however, completely abrogated FcR  $\gamma$ -chain-dependent functions in an Fc $\alpha$ RI R209L/M210R mutant. By coimmunoprecipitation, we have demonstrated the loss of a physical interaction between FcR  $\gamma$ -chain and Fc $\alpha$ RI M210R mutant, thus explaining the loss of FcR  $\gamma$ -chain-dependent functions. In conclusion, not only the presence of a basic residue in the transmembrane region of Fc $\alpha$ RI, but also the orientation of Fc $\alpha$ RI toward the FcR  $\gamma$ -chain dimer is essential for FcR  $\gamma$ -chain association. This suggests the involvement of additional amino acids in the Fc $\alpha$ RI-FcR  $\gamma$ -chain interaction. *The Journal of Immunology*, 2006, 176: 3603–3610.

The receptor for IgA, Fc $\alpha$ RI or CD89, is expressed on neutrophils, monocytes, eosinophils, macrophages, dendritic cells, and Kupffer cells (1–5). Fc $\alpha$ RI plays an important role in protection against infections. Activation of the receptor by IgA-immune complexes initiates numerous immune effector functions, including phagocytosis, oxidative burst, cytokine release, and degranulation (6–8). Fc $\alpha$ RI is also capable of Ab-dependent cell-mediated cytotoxicity in cooperation with complement receptor CR3 (9, 10). Fc $\alpha$ RI is not only important in host defense, but may also be valuable as a target for Ab therapy (11–13).

Fc $\alpha$ RI is a type I transmembrane receptor with two extracellular Ig-like domains, a hydrophobic transmembrane domain, and a short cytoplasmic tail (14, 15). For several functions, including calcium mobilization and cytokine production, the receptor critically depends on the associated FcR  $\gamma$ -chain dimer (16–18), although Fc $\alpha$ RI unassociated with the FcR  $\gamma$ -chain can retain some functions (19, 20).

The FcR  $\gamma$ -chain contains a so-called ITAM. Fc $\alpha$ RI ligation initiates ITAM phosphorylation, which mediates signaling further downstream (21–24). A stable complex of Fc $\alpha$ RI and FcR  $\gamma$ -chain is important for FcR  $\gamma$ -chain-dependent functions. This association is based on oppositely charged amino acids in the transmembrane region of both molecules (17).

Unlike other activating FcRs (Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\epsilon$ RI), a positively charged arginine at position 209 is unique for Fc $\alpha$ RI (25). This arginine at position 209 in the transmembrane region of Fc $\alpha$ RI can be replaced by a positively charged histidine without losing FcR  $\gamma$ -chain-dependent functions (17). It was recently shown that Fc $\alpha$ RI has an exclusive feature to exert a dual function: activatory function, shared by other FcRs, and an unique capacity to trigger inhibitory functions (26). Although Fc $\alpha$ RI is clearly an FcR, it is more related to members of the leukocyte receptor cluster (LRC),<sup>3</sup> including the leukocyte Ig-like receptors and killer Ig-like receptors, based on amino acid sequence identity (27, 28). Within the LRC family, an N-terminal positively charged arginine is highly conserved among all activatory receptors (27, 29), with the exception of the killer activatory receptors (KARs), which bear a positively charged lysine in the center of their transmembrane region (27). Interestingly, the KARs interact with DAP12 (30), whereas other LRC stimulatory receptors associate with the common FcR  $\gamma$ -chain (31), suggesting that a positive charge located at the N-terminal part is required for FcR  $\gamma$ -chain association. Furthermore, FcR  $\gamma$ -chain association of the other activating FcRs is based on nonbasic, polar residues in the C-terminal part of the transmembrane region (32).

Taken together, a basic amino acid in the transmembrane region of Fc $\alpha$ RI is essential for FcR  $\gamma$ -chain-dependent functions. Given

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Received for publication November 4, 2005. Accepted for publication January 4, 2006.

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<sup>1</sup> This work was supported by Dutch Cancer Foundation Grant UU 2002 2706 and Association for International Cancer Research Grant 03-119.

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<sup>3</sup> Abbreviations used in this paper: LRC, leukocyte receptor cluster; DAP, DNAX-associating protein; KAR, killer activatory receptors; Wt, wild type.

the conserved N-terminal position of the positively charged residue within the LRC family, we wondered whether optimal Fc $\alpha$ RI functioning requires this basic residue to be at position 209. To better define the nature of this interaction, Fc $\alpha$ RI mutants were generated in which the positive charge was relocated to new positions within the N-terminal part of its transmembrane region. Our data support the idea that the vertical position of this positively charged amino acid is crucial for functional assembly of the Fc $\alpha$ RI-FcR  $\gamma$ -chain complex, and these findings can possibly be extrapolated to other activating LRC members.

## Materials and Methods

### Generation of mutant Fc $\alpha$ RI cDNAs

The insertion of human Fc $\alpha$ RI cDNA into pCAV vector and murine FcR  $\gamma$ -chain cloned into pNUT vector were described previously (17). Construction of the Fc $\alpha$ RI R209L mutant was also described (17). The same strategy, based on overlap-extension PCR with mutated oligonucleotide primers, was used for the construction of both the Fc $\alpha$ RI R209L/A213H and the R209L/M210R mutant using Fc $\alpha$ RI-R209L cDNA as a template.

### Generation of stable transfectants

Generation of stable cell lines expressing the mutant Fc $\alpha$ R was performed as described previously (17). Briefly, the transfectants were made by electroporation of IIA1.6 cells with the mutant Fc $\alpha$ RI and the FcR  $\gamma$ -chain and subsequent selection with methotrexate (Pharmachemie); several subclones were tested in the described assays. The expression of Fc $\alpha$ RI was confirmed by flow cytometry, and FcR  $\gamma$ -chain was detected by Western blotting (see below).

### Cells

The murine CD5<sup>+</sup> B cell/macrophage cell line IIA1.6 (33) was cultured in RPMI 1640 (Invitrogen Life Technologies; 2 mM glutamine and 25 mM HEPES) supplemented with 10% heat-inactivated FCS (Integro), 100 U/ml penicillin (Invitrogen Life Technologies), and 100  $\mu$ g/ml streptomycin (Invitrogen Life Technologies). Fc $\alpha$ RI transfectants were maintained in the same medium supplemented with methotrexate (10  $\mu$ M).

### Flow cytometry

Fc $\alpha$ RI expression was measured by incubating the cells with mAb A59-PE (BD Pharmingen) or with an isotype control (mIgG<sub>1</sub>-RPE; DakoCytoma-

tion) for 30 min at 4°C. Cells were washed twice with PBS, 1% BSA, and 0.1% NaN<sub>3</sub> and were analyzed by FACSCalibur (BD Pharmingen).

### Western blotting

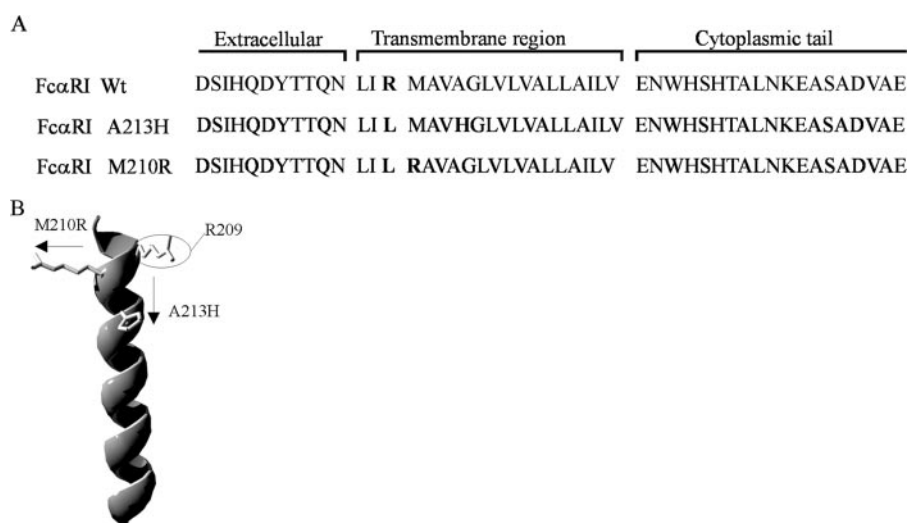
Total lysates of the transfectants were prepared in reducing Laemmli sample buffer and analyzed by SDS-PAGE on 15% polyacrylamide gels for detection of FcR  $\gamma$ -chain. The polyacrylamide gels were transferred on polyvinylidene difluoride membranes (Immobilon-P; Millipore), blocked with 5% low fat milk powder in PBS for 1 h at room temperature. For FcR  $\gamma$ -chain detection, the membranes were probed with a rabbit polyclonal anti-human  $\gamma$ -chain (1:1000; Upstate Biotechnology) for 1 h at room temperature, followed by incubation for 45 min with goat anti-rabbit IgG (H+L)-HRP (1:10,000; Pierce). After extensive washing in PBS and 0.05% Tween 20, bound Ab was detected by chemiluminescence (Amersham Biosciences).

### Ligand binding assay

As ligand, IgA-coated, 96-well plates were used to assess binding of the transfectants to IgA. Briefly, 96-well plates were coated overnight at 4°C with 100  $\mu$ l of human IgA serum (ITK Diagnostics; 10  $\mu$ g/ml) or 100  $\mu$ l BSA (10  $\mu$ g/ml; Roche) as a negative control. The next day, the plates were washed twice with PBS and 1% BSA. Cells ( $5 \times 10^6$ ) were prepared by incubation with 1  $\mu$ l of calcein-AM (20 mM; Molecular Probes) for 30 min at 37°C. After washing,  $2 \times 10^5$  cells were added per well of the coated plates. After incubation for 30–60 min at 4°C, plates were directly measured on a Fluoriscan (Molecular Probes) at 485 nm; this reading was designated input. Next, plates were washed to remove nonadherent cells until the fluorescence of the BSA-coated wells was reduced to the background level. Subsequently, adhesion of the transfectants to IgA was expressed as fluorescence after washing the plate divided by fluorescence before washing the plate (method adapted from Ref. 34). The ligand binding capacity of the Fc $\alpha$ RI wild-type (Wt) transfectant was set at 100%. The ligand binding capacity of the Fc $\alpha$ RI mutant receptor was calculated as a percentage compared with the Fc $\alpha$ RI Wt receptor. Data are shown as the mean  $\pm$  SD.

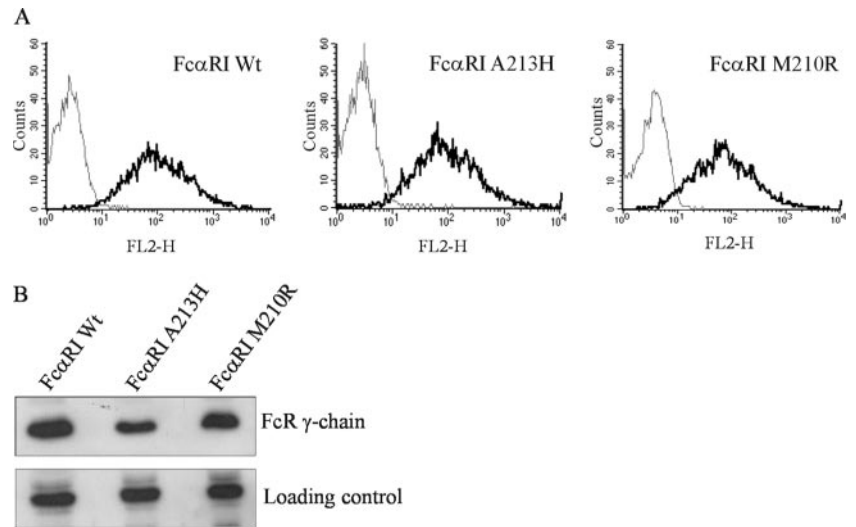
### Fc $\alpha$ RI internalization

Cells ( $2 \times 10^5$ ) were loaded with mAb anti-human CD89 or mIgG<sub>1</sub> isotype control for 60 min at 4°C. After washing, the cells were incubated with a second Ab, goat F(ab')<sub>2</sub> anti-mouse IgG1. At this point the samples were split in two. One sample was kept at 4°C to measure the total surface expression of Fc $\alpha$ RI, and the other sample was kept at 37°C for the indicated time periods. After these incubation periods, the surface Fc $\alpha$ RI expression was measured by staining the retained surface receptors with a

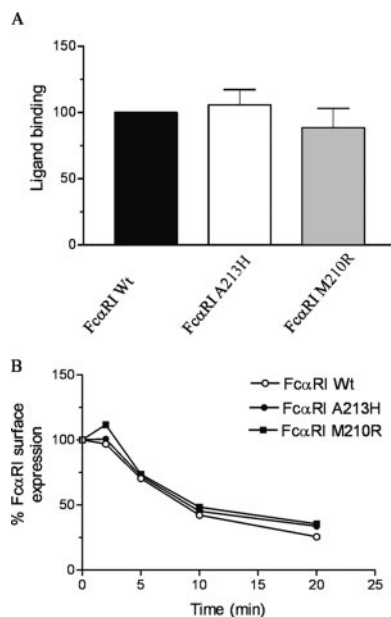


**FIGURE 1.** Schematic overview of Fc $\alpha$ RI Wt and Fc $\alpha$ RI mutant constructs. **A**, Amino acid sequence of Fc $\alpha$ RI (196–244). The positively charged arginine (R) at position 209 in the Fc $\alpha$ RI Wt transmembrane region is shown in bold. Within the transmembrane region of Fc $\alpha$ RI A213H mutant (in bold), R209 was replaced by an uncharged leucine (L), and the alanine at position 213 (A213) was replaced by a positively charged histidine (H). For the Fc $\alpha$ RI R209L/M210R mutant receptor, in the transmembrane region (in bold), R209 was replaced by an uncharged leucine (L), and methionine (M) at position 210 (M210) was replaced by a positively charged arginine (R). **B**, Helical model of the transmembrane region of Fc $\alpha$ RI. The R at position 209 (Fc $\alpha$ RI Wt) is circled. Lateral transfer of the positive charge to position 210 (Fc $\alpha$ RI M210R mutant) is indicated ( $\leftarrow$ ). The vertical relocation of the positive charge (Fc $\alpha$ RI A213H mutant) is marked ( $\downarrow$ ). Side chains of the amino acids R209, R210, and H213 are depicted.

**FIGURE 2.** Expression of Fc $\alpha$ RI and FcR  $\gamma$ -chain in IIA1.6 transfectants. *A*, Expression of Fc $\alpha$ RI in IIA1.6 Fc $\alpha$ RI transfectants ( $n > 3$ ). Cells were incubated with an anti-Fc $\alpha$ RI mAb (black line) or with mIgG<sub>1</sub>-PE (isotype control; gray line). *B*, Protein levels of the FcR  $\gamma$ -chain were measured in lysates of Fc $\alpha$ RI transfectants by Western blotting with an anti-FcR  $\gamma$ -chain antiserum. The expression of FcR  $\gamma$ -chain was shown by the presence of a 9-kDa protein band. Irrelevant protein bands, due to cross-reactivity of the secondary Ab staining, served as loading controls in all samples ( $n = 3$ ).



third Ab (R $\alpha$ G-IgG(H+L)-FITC conjugated; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. After washing, the samples were analyzed for Fc $\alpha$ RI surface expression on a FACSCalibur. Internalization of Fc $\alpha$ RI in the 37°C samples was calculated as a percentage of the total Fc $\alpha$ RI expression measured in the 4°C samples.



**FIGURE 3.** Ligand binding capacity and internalization of Fc $\alpha$ RI transfectants. *A*, Calcein-AM-labeled cells were incubated in 96-well plates that were coated with human IgA serum or BSA (negative control), and the fluorescence of the cells was measured at 485 nm on a Fluoriscan. Ligand binding was assessed by calculating the fluorescence of the transfectants after washing compared with the input. The ligand binding capacity of Fc $\alpha$ RI Wt was set at 100%. The percent ligand binding capacity compared with the Fc $\alpha$ RI Wt ( $n = 2$ ) is shown on the y-axis. Binding of all Fc $\alpha$ RI transfectants to BSA-coated plates remained  $<5\%$  (data not shown;  $n = 2$ ). *B*, To determine the internalization capacity of Fc $\alpha$ RI in the different transfectants, cells were loaded with an anti-human Fc $\alpha$ RI mAb or mIgG<sub>1</sub> (negative control) and subsequently with a goat F(ab')<sub>2</sub> anti-mouse IgG1. Half the samples were put on ice; the others were kept at 37°C for the indicated time periods (depicted on the x-axis). Next, retained surface receptors were stained with R $\alpha$ G-IgG (H+L)-FITC. Fc $\alpha$ RI internalization rates in 37°C samples were calculated as a percentage of the total Fc $\alpha$ RI expression measured in the 4°C samples (depicted on the y-axis;  $n = 3$ ).

### Calcium mobilization assay

Intracellular-free calcium levels were measured using cells simultaneously labeled with 1,5-(and-6)-carboxysemaphorhoda fluo 1-acetoxymethyl ester, acetate (2.8  $\mu$ M), and fluo 3-AM (1.4  $\mu$ M; Molecular Probes) by incubation for 30 min at 37°C in RPMI 1640 (2 mM glutamine and 25 mM HEPES) supplemented with 1% heat-inactivated FCS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Cells were washed and incubated with anti-human CD89 Ab or mIgG1 isotype control Ab, incubated for 30 min at 4°C, washed, and resuspended in RPMI 1640 (2 mM glutamine and 25 mM HEPES) supplemented with 1% FCS. Calcium mobilization was measured on a FACSCalibur. In the first 20 s, a baseline value for the intracellular-free calcium concentration was measured. Subsequently, Fc $\alpha$ RI-specific calcium mobilization was measured by adding 6  $\mu$ l of goat F(ab')<sub>2</sub> anti-mouse IgG1 (Southern Biotechnology). In parallel, for each cell line the release of calcium after BCR cross-linking was assessed by addition of goat F(ab')<sub>2</sub> anti-mouse IgG (Jackson ImmunoResearch Laboratories).

### MAPK phosphorylation assay

Cells ( $2 \times 10^5$ ) were incubated with anti-Fc $\alpha$ RI (A77) for 30 min at room temperature. After washing twice with RPMI 1640, F(ab')<sub>2</sub> of goat anti-mouse IgG1 Ab was added for the indicated time periods at 37°C to cross-link Fc $\alpha$ RI. Reactions were stopped by addition of 70  $\mu$ l of reducing SDS-PAGE sample buffer. Cross-linking of the BCR by addition of goat F(ab')<sub>2</sub> anti-mouse IgG for 2 min at 37°C served as a positive control. Background levels of MAPK phosphorylation were determined by omitting the cross-linking Ab or by omitting all Abs. Western blotting of these samples is described above. Membranes were blocked in 5% BSA (Roche) and probed with anti-phospho-p44/42 MAPK or anti-total MAPK Ab for 2 h (Cell Signaling Technology). After washing, membranes were incubated for 45 min with peroxidase-conjugated goat anti-rabbit Ab.

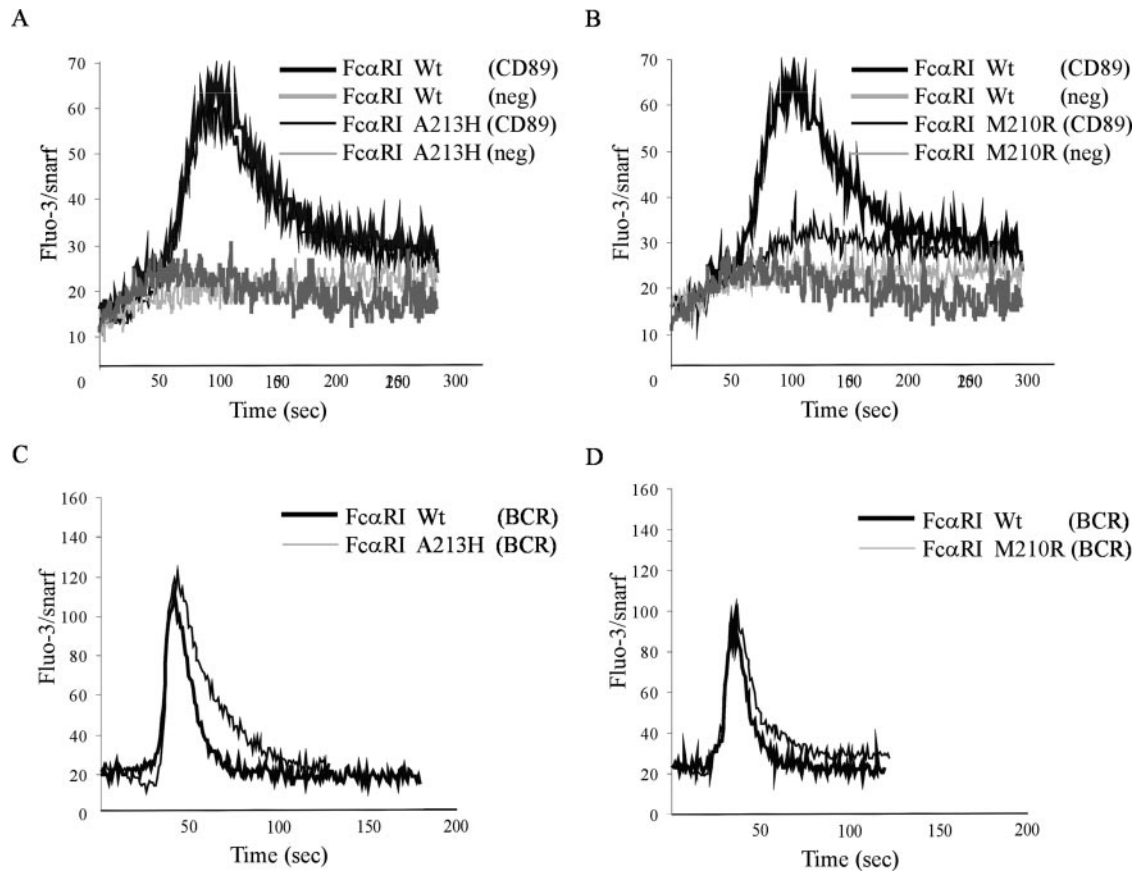
### IL-2 production

Transfectants were incubated for 30 min at 4°C with mAb anti-human CD89 or with mIgG1 isotype Ab as a control. After washing with PBS, goat F(ab')<sub>2</sub> anti-mouse IgG1 in complete medium was added to the cells and incubated overnight at 37°C. As a positive control, goat F(ab')<sub>2</sub> anti-mouse IgG was used to trigger the BCR. After overnight incubation, supernatants were isolated, and IL-2 production was measured by ELISA. For this purpose, a 96-well plate was coated overnight with the capture mAb, rat anti-mouse IL-2 (BD Pharmingen; 0.5 mg/ml) in 100  $\mu$ l of binding buffer (0.1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 9.0)). Subsequently, the plate was blocked with 100  $\mu$ l of 1% BSA in PBS for 1 h at room temperature, and 1/3 diluted samples were incubated for 2 h at room temperature. Next, a mixture of detecting Abs was added (biotin-rat anti-mouse IL-2 (BD Pharmingen) and streptavidin-peroxidase conjugated (Roche)). IL-2 production was measured at 405 nm on a MultiscanRC (Thermo LabSystems). Each supernatant was measured individually in the IL-2 ELISA.

### Coimmunoprecipitation

Protein A/G agarose beads (Santa Cruz Biotechnology) were incubated with 3  $\mu$ g of purified anti-human CD89 (0.5 mg/ml; BD Biosciences) or





**FIGURE 4.** FcR  $\gamma$ -chain dependent calcium release in Fc $\alpha$ RI transfectants. Calcium mobilization was triggered with an anti-human CD89 Ab (CD89) or mIgG<sub>1</sub> (neg) in 1,5-(and-6)-carboxysemaphorhoda fluor 1-acetoxymethyl ester/Fluo 3-loaded cells (A and B;  $n = 3$ ). In the first 20 s, a baseline value for the intracellular-free calcium concentration was measured. Subsequently, Fc $\alpha$ RI-initiated calcium mobilization was measured upon adding goat F(ab')<sub>2</sub> anti-mouse IgG1. BCR cross-linking (BCR) was assessed by addition of goat F(ab')<sub>2</sub> anti-mouse IgG (C and D;  $n = 2$ ). In every graph, the thick line marks the Fc $\alpha$ RI Wt cell line, and the thin lines represent Fc $\alpha$ RI mutant cell lines.

mIgG1 isotype control Ab (Sigma-Aldrich) in PBS and 1% BSA at 4°C for a minimum of 2 h for each immunoprecipitation. IIA1.6 cells ( $1 \times 10^7$  cells/immunoprecipitation) were collected by centrifugation and lysed in a digitonin lysis buffer (1% digitonin, 150 mM NaCl, 0.12% Triton X-100, and 20 mM triethanolamine) for 30 min at 4°C. The lysate was clarified by centrifugation at 15,000 rpm for 10 min at 4°C, and the supernatant was transferred to the Ab-coated beads. This mixture was incubated overnight at 4°C, then beads were isolated by centrifugation at 4,000 rpm for 10 min at 4°C and washed twice in digitonin lysis buffer. Precipitates were resuspended in Laemmli sample buffer and checked for FcR  $\gamma$ -chain by Western blotting.

## Results

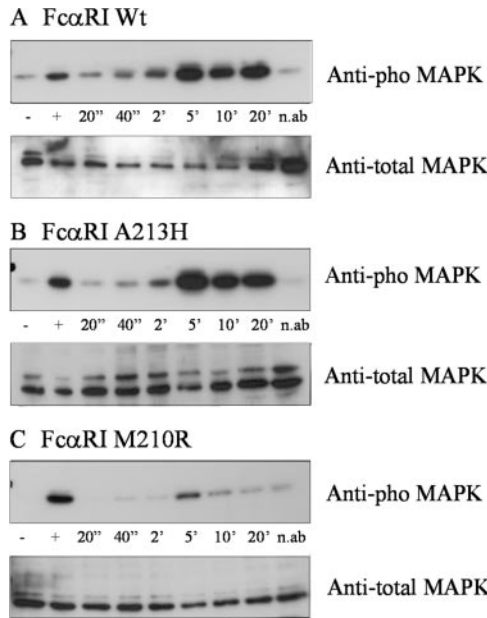
### Generation of Fc $\alpha$ RI transmembrane mutants

A functional association between Fc $\alpha$ RI and FcR  $\gamma$ -chain occurs when a positively charged residue (R209 or R209H) is present within the Fc $\alpha$ RI transmembrane domain, whereas a negative (R209D) or uncharged (R209L) amino acid at position 209 results in loss of Fc $\alpha$ RI functioning (17, 26). To investigate whether not only the presence of a positive charge, but also its localization in the transmembrane region of Fc $\alpha$ RI, is a prerequisite for functional FcR  $\gamma$ -chain association, we constructed two double mutants, Fc $\alpha$ RI R209L/M210R (M210R) and Fc $\alpha$ RI R209L/A213H (A213H; Fig. 1A). We relocated the positive charge from position 209 to either position 210 or 213 in the N-terminal part. We focused on the N-terminal region because more C-terminal-located positively charged amino acid would probably interact with DAP12 rather than FcR  $\gamma$ -chain (18, 27). The functionality of a

lateral or vertical relocation of a positive charge was determined in Fc $\alpha$ RI M210R and Fc $\alpha$ RI A213H (Fig. 1B). We decided to replace the methionine (M210) and the alanine (A213) with positively charged arginine and histidine, respectively, to minimally change the size of the amino acid side chains. Stable transfectants were generated by cotransfecting individual Fc $\alpha$ RI constructs with the murine FcR  $\gamma$ -chain in the murine IIA1.6 cell line. FACS analyses showed all transfectants to express similar levels of Fc $\alpha$ RI (Fig. 2A). Protein expression of FcR  $\gamma$ -chain was detected by Western blotting in the transfectants at similar levels (Fig. 2B).

### Fc $\alpha$ RI mutants mediate Fc $\alpha$ RI ligand binding and receptor internalization

To exclude the possibility of a conformational change in the extracellular Fc $\alpha$ RI part by introduction of mutations in the transmembrane region of the Fc $\alpha$ R and differences in the activation state of the receptor (35), we tested ligand binding and internalization of Fc $\alpha$ RI. First, the Fc $\alpha$ RI ligand binding capacity was determined using plates coated with human serum IgA and fluorescence-labeled cells (34). Both Fc $\alpha$ RI A213H and Fc $\alpha$ RI M210R cells bound to IgA-coated plates with similar capacity as Fc $\alpha$ RI Wt (Fig. 3A). Second, we examined Fc $\alpha$ RI internalization upon receptor cross-linking. As shown in Fig. 3B, Fc $\alpha$ RI Wt and Fc $\alpha$ RI mutants internalized with similar kinetics. These data demonstrate that both Fc $\alpha$ RI mutants retain intact  $\alpha$ -chain properties.



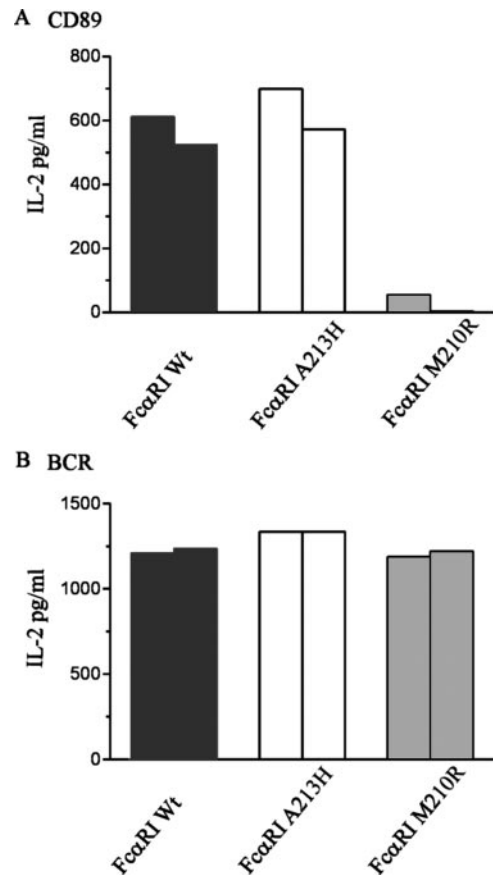
**FIGURE 5.** Fc $\alpha$ RI cross-linking triggered MAPK phosphorylation. Cells were incubated with an anti-Fc $\alpha$ RI mAb and cross-linked with goat F(ab')<sub>2</sub> anti-mouse IgG1 for 20 s (20'') to 20 min (20') to induce Fc $\alpha$ RI-mediated MAPK phosphorylation. As a negative control, cells were only incubated with anti-Fc $\alpha$ RI mAb (–) or no Ab (n.ab). As a positive control, cells were incubated with goat F(ab')<sub>2</sub> anti-mouse IgG for 2' to trigger the BCR (+). For each cell line, anti-phospho-MAPK staining is shown in the upper panels, and total MAPK is shown in the lower panels ( $n = 2$ ).

#### Fc $\alpha$ RI M210R mutant signaling is abrogated

Fc $\alpha$ RI cross-linking is known to trigger calcium release from intracellular stores (36). To test Fc $\alpha$ RI functioning after relocation of the positive charge, we assessed the ability of both Fc $\alpha$ RI mutants to trigger intracellular calcium release. We measured Fc $\alpha$ RI-specific calcium flux after cross-linking with anti-Fc $\alpha$ RI mAb (A59) and goat anti-mouse IgG1 (Fig. 4, A and B). Using an isotype control mIgG<sub>1</sub> Ab and the secondary Ab, goat anti-mouse IgG1 (negative), did not result in a calcium flux. The Fc $\alpha$ RI Wt transfectant showed a maximum calcium flux at 100 s (Fig. 4A). By triggering the Fc $\alpha$ RI A213H mutant, we observed a Wt-like phenotype (Fig. 4A). In contrast, the Fc $\alpha$ RI M210R mutant was severely hampered in calcium mobilization upon Fc $\alpha$ RI triggering (Fig. 4B). IIA1.6 cells endogenously express the BCR (surface IgG2a) (37). All Fc $\alpha$ RI transfectants expressed BCRs at similar levels (data not shown;  $n = 2$ ). As shown in Fig. 4, C and D, all transfectants were capable of calcium mobilization upon BCR triggering.

Because calcium mobilization is a PI3K-mediated pathway and triggering Fc $\alpha$ RI can also induce RAS/MAPK kinase/MAPK activation, eventually leading to activation of several Fc $\alpha$ RI functions (7, 13, 35, 38, 39), we next investigated MAPK phosphorylation upon Fc $\alpha$ RI cross-linking. The Fc $\alpha$ RI Wt (Fig. 5A) and the Fc $\alpha$ RI A213H (Fig. 5B) mutants induced MAPK phosphorylation at 5 min, which lasted until at least 20 min, whereas MAPK phosphorylation for the Fc $\alpha$ RI M210R (Fig. 5C) mutant was less prominent and faded quickly. BCR triggering induced MAPK phosphorylation, measured at 2 min.

A more distal function triggered by Fc $\alpha$ RI is IL-2 production (17). For all transfectants, IL-2 secretion was triggered by Fc $\alpha$ RI cross-linking. As shown in Fig. 6A (data shown in duplicate), Fc $\alpha$ RI Wt and Fc $\alpha$ RI A213H were capable of producing significant levels of IL-2. In contrast, the Fc $\alpha$ RI M210R mutant dis-



**FIGURE 6.** Fc $\alpha$ RI triggered IL-2 release. IL-2 production was measured with an IL-2 ELISA in supernatants after overnight stimulation following Fc $\alpha$ RI cross-linking in the different transfectants (A) or the BCR (B). ■, IL-2 production of Fc $\alpha$ RI Wt; □, IL-2 production of Fc $\alpha$ RI A213H; ▤, IL-2 production of Fc $\alpha$ RI M210R. Each experiment was performed in duplicate. Bars are representative of three independent experiments. No IL-2 release was measured using an isotype control Ab ( $n = 3$ ; data not shown).

played a reduced capacity to produce IL-2 upon Fc $\alpha$ RI stimulation. No IL-2 release was measured using an isotype control Ab (data not shown). BCR triggering resulted in normal IL-2 production for all transfectants (Fig. 6B).

#### Fc $\alpha$ RI M210R does not associate with FcR $\gamma$ -chain

Because calcium mobilization, MAPK phosphorylation, and IL-2 production are dependent on interaction between Fc $\alpha$ RI and FcR  $\gamma$ -chain, we next tested this interaction in the Fc $\alpha$ RI mutants. Coimmunoprecipitations were performed for each cell line with Fc $\alpha$ RI specific-A59 Ab and mIgG<sub>1</sub> as an isotype control. Coimmunoprecipitation of FcR  $\gamma$ -chain was comparable for Fc $\alpha$ RI Wt and Fc $\alpha$ RI A213H (Fig. 7). The Fc $\alpha$ RI M210R mutant, however, did not coimmunoprecipitate FcR  $\gamma$ -chain. No immunoprecipitation was detected using isotype control Ab. Besides loss of interaction in this stably transfected cell line, we confirmed the inability of the Fc $\alpha$ RI M210R mutant to bind the FcR  $\gamma$ -chain in a transient transfection system in HEK 293T cells (data not shown). Fc $\alpha$ RI Wt, however, was fully capable of FcR  $\gamma$ -chain association under these conditions. This suggests that the physical interaction between the Fc $\alpha$ RI M210R mutant and the FcR  $\gamma$ -chain is disrupted.

## Discussion

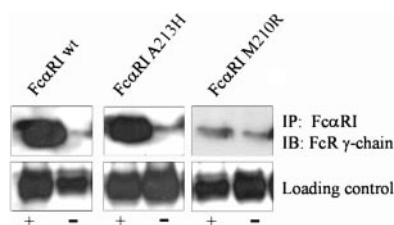
Similar to activating Fc $\gamma$ R, the Fc $\alpha$ R forms a complex with an FcR  $\gamma$ -chain homodimer in myeloid cells (17, 40, 41). However, unlike

Fc $\gamma$ R, the molecular basis of this interaction involves a positively charged arginine residue (R) at position 209 in the transmembrane domain of Fc $\alpha$ RI and a negatively charged aspartic acid (D) in the FcR  $\gamma$ -chain transmembrane domain (17). We studied the influence of the position of the positive charge in the transmembrane region of Fc $\alpha$ RI in two Fc $\alpha$ RI transmembrane mutants, thereby testing the spatial requirements for FcR  $\gamma$ -chain association. A vertical relocation of the positive charge in the  $\alpha$ -helical transmembrane of Fc $\alpha$ RI was realized in the Fc $\alpha$ RI A213H mutant. The Fc $\alpha$ RI M210R mutant represents a lateral transfer of the positive charge. These Fc $\alpha$ RI mutants were tested for FcR  $\gamma$ -chain-dependent and -independent functions and compared with the Fc $\alpha$ RI Wt.

Although it is difficult to exclude subtle conformational changes in the Fc $\alpha$ R after introducing point mutations in its transmembrane region, no indications of conformational changes were obtained after replacement of the arginine by a leucine (R209L), as shown previously (17). By substitution of the methionine, and alanine for a positively charged arginine (R) and histidine (H), respectively, we changed the amino acids to a minimal extent; in addition, the arginine and histidine were interchangeable without affecting Fc $\alpha$ RI functioning, as previously described (17). We can exclude major conformational changes of the  $\alpha$ -chain because we found Fc $\alpha$ RI surface expression (Fig. 2A), normal IgA binding ability of the Fc $\alpha$ RI mutants, and the capacity to internalize Fc $\alpha$ RI in both mutant receptors (Fig. 3).

In the present report, we found that the positive charge can be relocated within the transmembrane region of Fc $\alpha$ RI without losing FcR  $\gamma$ -chain-dependent functions, as shown for the Fc $\alpha$ RI A213H mutant. Lateral relocation, however, of the positive charge in the Fc $\alpha$ RI M210R mutant had a major impact on FcR  $\gamma$ -chain-dependent functionality. After lateral translocation, no FcR  $\gamma$ -chain association was found under mild lysis conditions (Fig. 7), resulting in the loss of signaling via the PI3K and Ras-ERK pathways. This was shown for calcium mobilization (Fig. 4), MAPK phosphorylation (Fig. 5), and IL-2 production (Fig. 6). Although Fc $\alpha$ RI transgenic mice are dependent on the FcR  $\gamma$ -chain for Fc $\alpha$ RI surface expression (9), a number of other studies showed Fc $\alpha$ RI surface expression not to be dependent on FcR  $\gamma$ -chain association (17, 19, 20), in line with our findings for the Fc $\alpha$ RI M210R mutant, which expressed Fc $\alpha$ RI at similar levels as Fc $\alpha$ RI Wt.

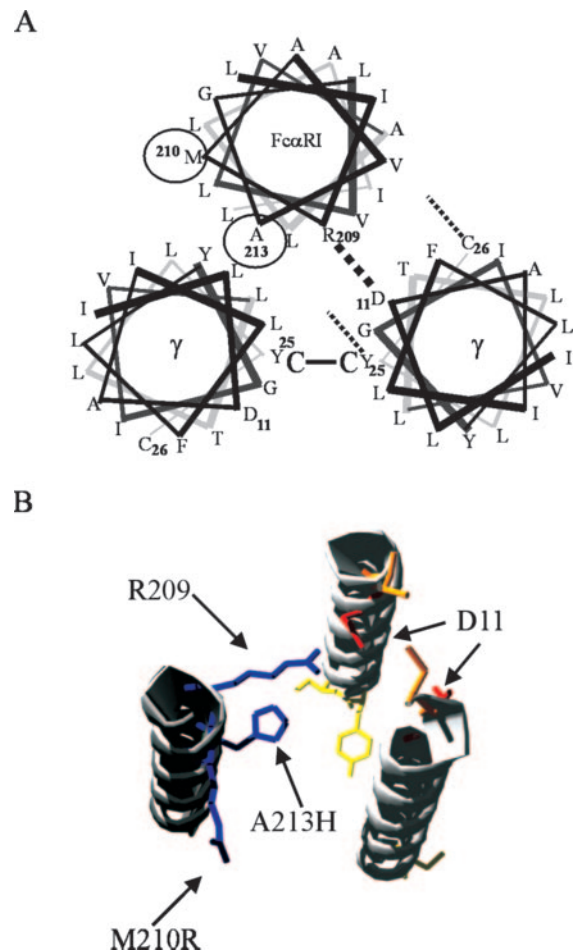
Because the crystal structure of Fc $\alpha$ RI has only been elucidated for the extracellular part of the receptor (42, 43), we propose a model to clarify the vertical and lateral relocations of the positive charge and its outcome on Fc $\alpha$ RI functioning. The transmembrane sequence was tested in different prediction programs, resulting in



**FIGURE 7.** Physical interaction between Fc $\alpha$ RI and FcR  $\gamma$ -chain. Cells ( $1 \times 10^7$ ) were lysed in a digitonin-containing lysis buffer, and supernatants were transferred to anti-human Fc $\alpha$ RI-coated beads or mIgG<sub>1</sub> (isotype control)-coated beads. Mixtures were incubated overnight at 4°C. Next, beads were isolated, and precipitates were resuspended in Laemmli sample buffer and checked for FcR  $\gamma$ -chain. +, Fc $\alpha$ RI-specific Co-IP; -, Co-IP via isotype control. Irrelevant protein bands, due to cross-reactivity of the secondary Ab staining, served as loading controls in all samples. Three experiments were performed, yielding essentially identical results.

the same  $\alpha$ -helical wheel model (Fig. 8A) (44, 45). In this model, R209 and H213 in the Fc $\alpha$ RI A213H both face the FcR  $\gamma$ -chain, whereas M210R turns sideways (Fig. 8B).

The functional capabilities of the Fc $\alpha$ RI A213H support this model; i.e., this mutant receptor probably associates with FcR  $\gamma$ -chain in the same orientation as Fc $\alpha$ RI Wt, parallel to the original arginine to aspartic acid interaction. Relocation of the positive charge laterally in the Fc $\alpha$ RI M210R (Fig. 8A) results in transfer of the positive charge outside the interface of Fc $\alpha$ RI and FcR  $\gamma$ -chains, although still N-terminally and in close proximity with the original positive charge (Figs. 1 and 8B). This resulted in loss of FcR  $\gamma$ -chain-dependent functions due to an inability to assemble with the FcR  $\gamma$ -chain. Several factors may underlie these data. First, as recently described (18), not a single  $\gamma$ -chain, but dimerization of two  $\gamma$ -chains, is essential for Fc $\alpha$ RI assembly, resulting



**FIGURE 8.** Schematic model of Fc $\alpha$ RI-FcR  $\gamma$ -chain association. *A*, Schematic diagram of the transmembrane region of Fc $\alpha$ RI (aa 207–224) interacting with the transmembrane regions of human FcR  $\gamma$ -chain homodimer (aa 9–26). Predicted helical wheel diagrams are shown ([www.site.uottawa.ca/~turtocotte/resources/HelixWheel](http://www.site.uottawa.ca/~turtocotte/resources/HelixWheel)). The N termini of the transmembrane regions start with a thick black line and end with a thin gray one (C-terminal end of the transmembrane regions). The association between the arginine (R) at position 209 in Fc $\alpha$ RI and the aspartic acid (D) at position 11 in FcR  $\gamma$ -chain is marked by a thick dotted line. The FcR  $\gamma$ -chain transmembrane disulfide bond between cysteines (C) is marked by a solid line. The positions of A213 and M210 are marked by circles. The additional Fc $\alpha$ RI-interacting amino acids (Y25 and C26) of the FcR  $\gamma$ -chain are represented by thin dotted lines. A three-dimensional figure is depicted in *B*. Depicted are the side chains of the amino acids R209, H213, and R210 of Fc $\alpha$ RI (in blue), the D11 of FcR  $\gamma$ -chain (in red), extracellular cysteines (in orange), and the Y26 and C26 (in yellow).



in a three-helical interface. Second, the cysteines that form the disulfide bond between the two FcR  $\gamma$ -chains may also contribute to the assembly of all three subunits. Third, recent observations (44) demonstrated that in addition to the aspartic acid at position 11 within the FcR  $\gamma$ -chain, a tyrosine at position 25 (Y<sub>25</sub>) and a cysteine at position 26 (C<sub>26</sub>) contribute to stabilization of the Fc $\alpha$ RI-FcR  $\gamma$ -chain complex. Which Fc $\alpha$ RI amino acids are involved in these interactions is unknown. However, by replacing the methionine at position 210 with an arginine, we cannot exclude the possibility of disrupting an unknown interaction between methionine and FcR  $\gamma$ -chain.

Taken together, the assembly of Fc $\alpha$ RI and FcR  $\gamma$ -chain is not exclusively based on the arginine to aspartic acid interaction. Several other residues play an essential role in the formation of the three-helical interface. Replacement of the positive charge outside this interface could result in disruption or inadequate formation of the Fc $\alpha$ RI-FcR  $\gamma$ -chain complex. Notably, the importance of the position of a positive charge for complex formation has also been described for TCR-CD3 assembly (46). The  $\alpha$ -chain of the TCR bears two positive residues on opposite sides, allowing association with both CD3 $\delta$ - and CD3 $\epsilon$ -chains. Relocation of one of the positive charges abrogated association of the  $\alpha$ -chain with the CD3 $\delta\epsilon$  heterodimer, which coincided with the lack of formation of the TCR  $\alpha\beta$  complex.

From previous studies of FcRs, it is known that polar transmembrane residues are required for FcR  $\gamma$ -chain association for optimal receptor functioning, indicating that other residues can interact with the FcR  $\gamma$ -chain. For Fc $\gamma$ RI, the last 10 aa within the transmembrane region of Fc $\gamma$ RI, including the polar asparagine, are important for FcR  $\gamma$ -chain-dependent phagocytosis (41, 47). In Fc $\epsilon$ RI and Fc $\gamma$ RIIIa, a negatively charged aspartic acid is present in the transmembrane region and mediates FcR  $\gamma$ -chain association and functioning (48–50). Replacement of the transmembrane region of the FcR  $\gamma$ -chain with the CD8 transmembrane region (32) abrogated phagocytosis, indicating that the transmembrane part is crucial for association.

In summary, this present study further defines the molecular basis for Fc $\alpha$ RI-FcR  $\gamma$ -chain association. Vertical positioning of the basic residue is found to be indispensable within the three-helical interface for functional assembly of the Fc $\alpha$ RI-FcR  $\gamma$ -chain dimer complex. This implies that multiple interactions contribute to stable Fc $\alpha$ RI-FcR  $\gamma$ -chain complexes.

## Acknowledgments

We thank Prof. P. J. Coffey for critically reading this manuscript.

## Disclosures

The authors have no financial conflict of interest.

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