Tyrosine-containing Sequence Motifs of the Human Immunoglobulin G Receptors FcRIIb1 and FcRIIb2 Essential for Endocytosis and Regulation of Calcium Flux in B Cells

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Human B cells express two closely related immunoglobulin G receptors, FcRIIb1 and FcRIIb2, which differ by a 19 amino acid insertion in the cytoplasmic tail of FcRIIb1. The cytoplasmic tails of both isoforms contain a conserved sequence motif (AENTITYSLL) essential for mediating endocytosis via FcRIIb2. Truncation of this motif abolished endocytosis, while replacement of tyrosine (Ty)-273 in FcRIIb2 by phenylalanine had no effect on the amount and kinetics of ligand uptake. Co-cross-linking of FcRIIb1 or FcRIIb2 with the antigen receptor on B cells led to an abortive calcium signal. Neither isoform interfered with the early intracellular calcium mobilization, but both prevented the opening of a plasma membrane calcium channel essential for a sustained elevated intracellular calcium level. Modulation of calcium channel activity is mediated by the same sequence motif essential for endocytosis but requires the presence of Ty-292 in FcRIIb1 and Ty-273 in FcRIIb2. Co-cross-linking of FcRIIb1 with surface IgG is associated with tyrosine phosphorylation of Ty-292, whereas Ty-273 in FcRIIb2 was not phosphorylated. Thus, FcRIIb phosphorylation is probably not directly involved in the modulation of the calcium signal but may be essential for further diversification of signals induced via the co-expressed isoforms FcRIIb1 and FcRIIb2.

Immunoglobulin G receptors (FcR)1 exert a suppressive signal on the antigen-induced B cell differentiation when co-cross-linked with sIg on B cells (1). Engagement of the FcR prevents the sustained elevated intracellular calcium level in response to sIg cross-linking (2, 3). Therefore, the plasma membrane calcium channel has been implicated to be the target of the FcR-derived negative signal. The mechanism by which the FcR interferes with the opening of the calcium channel is elusive. Signal transduction events that mediate calcium signaling in B cells involve the activation of protein tyrosine kinases to the antigen receptor complex (4–7). Antigen receptor clustering leads to tyrosine phosphorylation of Ig-α and Ig-β as well as PLC-γ1/2 (4, 8), which in turn generates inositol trisphosphate thereby liberating calcium from intracellular stores (4, 5). Recent studies revealed that Ig-α mainly accounts for the calcium signal elicited after antigen receptor cross-linking although clustering of both chains led to enhanced tyrosine phosphorylation (9).

The characterization of the biochemical events leading to the inactivation of the plasma membrane calcium channel has been further complicated by the finding that human B cells, in contrast to mouse B cells, can express all FcRI (CD32) isoforms (FcRIa, c, b1 and b2; 10, 11, for a recent review see Ref. 12). The signals mediated by ARH1 motif containing FcRIa/FcRIc isoforms in B cells have been studied recently in our laboratory (13). The FcRIb isoforms which are the predominantly expressed FcR in human B cells reside from alternative splicing and differ by a 19 amino acid insertion in the cytoplasmic tail of FcRIIb1. Since human B cells in contrast to mouse B cells (FcRIIb1/FcRIIb2) express both isoforms we aimed to determine whether both human FcRIb isoforms generate redundant or divergent intracellular signals when expressed as a sole FcR in the FcR+ mouse B cell line IL41.6. By generating cytoplasmic truncated FcRIIb2 mutants and by site-directed mutagenesis of structurally related tyrosine residues present in both isoforms, we found that a sequence motif (AENTITYSLL) conserved between mouse and human FcRIIb isoforms mediates calcium flux regulation and endocytosis of small IgG complexes. Here we show that the homologous tyrosine residues within the conserved sequence motif are essential for the down-regulation of the plasma membrane calcium channel. Co-cross-linking of FcRIIb1, but not of FcRIIb2, with sIgG is associated with FcR tyrosine phosphorylation.

MATERIALS AND METHODS

Generation of Receptor Mutants—The cDNA of FcRIIb2 was isolated from a placental cDNA library as described recently (14). The cDNA of FcRIIb1 was generated by reverse transcriptase-polymerase chain reaction using the FcRIIb2 cDNA as a template. Briefly, a 392-base pair BamHI/RgII fragment of PL17 cloned in pUC18 was chosen as a template. The numbering refers to M1 and M13 have already been described (16). The numbering refers to the respective positions on the PCR products and generation of the complete FcRIIb1 cDNA. The FcRIa-HR cDNA has been cloned via reverse transcriptase-polymerase chain reaction from total RNA prepared from leukocytes of a systemic lupus erythematosus patient (15). The FcRIIb2 cytoplasmic deletion mutants M1 and M13 have already been described (16). The numbering refers to the number of amino acids within the cytoplasmic region of the mutants. FcRIIb2 mutants M25 and M35 were obtained by introducing stop codons at the respective positions of the cytoplasmic tail (16). Briefly, a 392-base pair EcoRl/RgII fragment cloned in M13mp18 was used as a template for PCR. The DNA was amplified using M13 reverse primers and the mutagenic oligonucleotide containing either a BamHI stop codon.

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2 The abbreviations used are: FcR, immunoglobulin G receptor; sIg, surface Ig; ahlgG, heat-aggregated human IgG; ARH1, antigen receptor homology 1 motif; Ig, immunoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PLC, phospholipase C; RAM, intact rabbit anti-mouse IgG antibodies; wt, wild type(s); FTTC, fluorescein isothiocyanate.
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(M35, 5'-GCTCTGGAGGATCCTGAGC-3') or a SpH1 (M25, 5'-CAC-
CTAACATCTAACTGCA-3') restriction site, which allowed further
subcloning of the mutant. The tyrosine residues in the cytoplasmic
region of FcRIIb and FcRIIb2 were modified into a phenylalanine
using the U.S.E. in vitro mutagenesis system as recommended by the
supplier (Pharmacia). Mutagenic clones were generated using the
U.S.E. MutII/Sacl selection primer converting a ScaI restriction enzyme
site in pUC18 into a MutII site allowing selection against wild type
phenylalanine. In addition the mutagenic oligonucleotides (FcRIIb-
FrclbFrclb2-P273; 5'-CACTAATCTAATCTGCA-3') and
FcRllbFrclb-fl58; 5'-GCTCTCCGGATCTCCTG-3') contained a
newly introduced restriction enzyme site to facilitate screening of
the clones. All new inserted restriction enzymes were verified by sequencing both strands by the dyeodeoxy chain termination method (17).

Qualitative and Quantitative Measurement of FcRII-mediated ahIgG
Internalization—To determine the uptake of ligand by immunofluores-
cence microscopy FcRII expressing IIA1.6 cells were processed as
described in detail recently (13). Cells were incubated for 30 min at 4°C
with 20 pg/ml ahIgG (14). After extensive washing the cells were at-
tached to multwell coverslips. Endocytosis of bound ligand was initia-
ted by incubating the cells for 30 min at 37°C. Uptake was stopped by
chilling the cells on ice, while the control cells were constantly kept
on ice. After fixation using PBS containing 3.7% formaldehyde and 2%
sucrose for 15 min at 4°C, the cells were permeabilized with PBS
supplemented with 0.2% Triton X-100 for 3 min at 20°C. Endocytosis
was visualized using an F(ab'), fragment of FITC-conjugated goat anti-
human IgG (Dianova, Hamburg, Germany) (21). The adherent cells were recovered after trypsin (Biochrom KG, Berlin, Germany) treatment
for 5 min. Immunoperoxidase staining was done using rabbit anti-phosphotyrosine antibodies (Biomol, Hamburg, Germany). After extensive washing bound antibodies were detected
by incubation in PBS, 3% skimmed milk for 1 h. Filters were probed with
1 pgiml anti-phosphotyrosine antibody 4G10 (Biomol, Hamburg, Germany) for 2 h in PBS, 3% skimmed milk and subsequently with horseradish
peroxidase-labeled goat anti-mouse IgG + IgM (Dianova, Hamburg, Germany) for an additional 90 min. To exclude that the lack of detectable phospho-
ylation of FcRIIb2 is a result of the mAb 4G10 used, we additionally used rabbit anti-phosphotyrosine antibodies (Biocorn, Hamburg, Germany). After extensive washing bound antibodies were detected
by incubation in PBS, 3% skimmed milk and subsequently with horseradish
peroxidase-labeled goat anti-mouse IgG + IgM (Dianova, Hamburg, Germany) for an additional 90 min.

RESULTS

The FcRllb1 and FcRllb2 Possess Different Capacities to Mediate
Endocytosis in B Cells Distinct from FcRlla—In this study we determined the endocytosis efficiency of the FcRllb isoforms b1 and b2 expressed in B cells in contrast to the
FcRlla isoform. We extended our previous results (13) obtained for the low responder allelic variant of FcRlla (FcRlla-HR) with the high responder variant of FcRlla (FcRlla-HR). This polymorphism has been originally defined by their different affinity for murine IgGl immune complexes, which is a result of an amino acid substitution at position 131 (arginine in FcRlla-HR instead of histidine in FcRlla-LR) (26). As determined by immuno-
fluorescence microscopy, FcRllb2 expressing cells internalize most of the bound ahIgG within 30 min (Fig. 1B), while FcRllb1-expressing cells clearly showed no intra-cellular staining (Fig. 1G). In contrast to FcRllb2 we observed most of the ligand clustered in polar caps, which were undetectable at 4°C.

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Endocytosis of anti-IgG by FcRIIb2-expressing B Cells Is Dependent on a Region between Amino Acids 25 and 35 of the Cytoplasmic Domain—By introducing stop codons into the cDNA of FcRIIb2, we generated a series of receptors with truncated cytoplasmic tails (Fig. 4). Two of the deletion mutants, FcRIIb2-M25 and FcRIIb2-M35, were designed to analyze the function of a motif present in the FcRIIb isoforms (AENTILYSLL). This motif nearly resembles that found in other endocytic receptors as the manganese 6-phosphate receptor, the transferrin receptor, or the low density lipoprotein receptor, consisting of polar and positively charged amino acids proximal to a tyrosine residue (27). To analyze the functional importance of the tyrosine residue within this motif, we introduced a phenylalanine residue at position 273 in FcRIIb2 and position 292 in FcRIIb1 (Fig. 4). The tyrosine located at position 258 in the intervening 19 amino acids of the cytoplasmic tail of FcRIIb1 was replaced by a phenylalanine to analyze its possible role in blocking immune complex internalization (Fig. 4).

As determined by immunofluorescence microscopy, FcRIIb2-M35 lacking the carboxyl-terminal 9 amino acids internalizes anti-IgG comparable to the FcRIIb2 wt (Fig. 1C). In contrast, FcRIIb2-M25, which lacks amino acids YSLL of the motif, only revealed cell surface staining after 30 min of incubation at 37 °C (Fig. 1D). Similarly, mutants M13 and M1 were ineffective in triggering the uptake of anti-IgG (data not shown). Replacing Tyr273 in FcRIIb2 by phenylalanine (FcRIIb2-F273) did not influence the endocytosis efficiency (Fig. 1E). Additionally, the FcRIIb1-F292 and FcRIIb1-F258 mutants were as ineffective as the FcRIIb1 wt in mediating endocytosis of small immune complexes (Fig. 1, G-I). Obviously, amino acids other than Tyr258 within the 19 amino acid insertion of FcRIIb1, which blocks endocytosis very efficiently, must be essential (Fig. 1G).

To evaluate whether the deletion or mutation of amino acids in the cytoplasmic tail of FcRIIb2 might influence both the amount and kinetics of endocytosis, the transfectants were analyzed by flow cytometry. As summarized in Fig. 3B, the amount and the kinetics of anti-IgG uptake of M35 were nearly indistinguishable from that of FcRIIb2 wt. Additionally, replacement of Tyr273 by a phenylalanine residue affected the endocytosis of bound anti-IgG only marginally. The small difference observed varied up to 5% among three different selected cell clones analyzed. Using the more sensitive method of flow cytometry compared to immunofluorescence microscopy, we could not detect internalization of anti-IgG either bound to FcRIIb1 wt or the (Y→F) mutants of FcRIIb1 (Fig. 3B).

Similar results were obtained for the mouse FcRIIb2 isoform, but alanine instead of tyrosine significantly reduced the amount of internalized ligand (28, 29). Our results show that, although endocytosis via FcRIIb2 is dependent on the presence of the tyrosine containing sequence motif, the respective tyrosine residue is irrelevant for endocytosis. Therefore, our results strongly favor a di-leucine containing endocytosis motif which is part of this homologous sequence and deleted in FcRIIb2-M25.

Cross-linking of FcRIIb1 or FcRIIb2 on B Cells Does Not Trigger Calcium Signaling and Tyrosine Phosphorylation of Fc—We have recently shown that in FcRIIa-LR expressing IIA16 cells, anti-IgG binding induces a transient rise in the intracellular calcium concentration independent of extracellular calcium (13). This is due to a signal transducing motif within the cytoplasmic domain of both allelic FcRIIa variants (Fig. 4). In contrast, FcRIIb1 or FcRIIb2 cross-linking did not lead to an increase in the intracellular calcium concentration (Fig. 5, A and B). Furthermore, FcRIIb1 or FcRIIb2 cross-linking by anti-IgG did not cause tyrosine phosphorylation of FcRIIb1 and b2 (Fig. 6), whereas FcRIIa became rapidly phosphorylated (13). Thus, the minimal protein tyrosine kinase recognition

Fig. 1. Binding and endocytosis of anti-IgG by FcRIIb1, FcRIIb2 wt, and receptor mutants expressed in the mouse B cell line IIA16. Cells were incubated with 20 μg/ml anti-IgG at 4 °C and then allowed to internalize the ligand for 30 min at 37 °C (B-E and G-I). Cell surface-bound and internalized ligand was visualized after permeabilization and incubation with the FITC-labeled F(ab′)2 fragment of goat anti-human IgG. FcRIIb1 (F) and FcRIIb2 (A) wt were maintained on ice to determine cell surface-bound anti-IgG. A, FcRIIb2 wt 4 °C; B, FcRIIb2 wt 37 °C; C, FcRIIb2-M35 37 °C; D, FcRIIb2-M25 37 °C; E, FcRIIb2-F273 37 °C; F, FcRIIb1 wt 4 °C; G, FcRIIb1 wt 37 °C; H, FcRIIb1-F258 37 °C; I, FcRIIb1-F292 37 °C. Bar, 10 μm.

Their appearance therefore reveals the expected temperature dependence (Fig. 1F).

Cell clones expressing equal numbers of FcRIIa-HR, FcRIIb2, and FcRIIb1 were selected to evaluate the amount and kinetics of FcRIIb2-mediated uptake (Fig. 2). Endocytosis of bound anti-IgG was assessed by flow cytometry and expressed as the difference between maximum binding at 4 °C and remaining cell surface-bound ligand at 37 °C. As summarized in Fig. 3A, FcRIIa-HR internalizes anti-IgG very rapidly, comparable to FcRIIa-LR (13). Within 5 min a plateau was reached corresponding to 50% endocytosis of the originally bound ligand. Compared to FcRIIa, FcRIIb2 mediated a slow uptake of anti-IgG, with no clearly detectable maximum (Fig. 3A). After 5 min at 37 °C, only 13% of the FcRIIb2-bound anti-IgG had been internalized, which is about one-third of that observed for FcRIIa. Further incubation of FcRIIb2 expressing IIA16 cells for up to 30 min increased the amount of internalized ligand to 33%, which is significantly lower than for FcRIIa (50%). Although both FcRIIa and FcRIIb2 mediated the endocytosis of anti-IgG in B cells, the type of Fc expressed has pronounced effects on the rate and the amount of internalized ligand which is in contrast to our previous results obtained for FcRIIa and FcRIIb2 expressed in the FeR fibroblastic cell line BHK-21 (16). In these cells FcRIIa and FcRIIb2 revealed no difference in the amount of internalized ligand, which might be due to cell type-specific associated effector proteins. FcRIIb1, which differs from FcRIIb2 by a 19 amino acid insertion within the cytoplasmic tail, failed to mediate the internalization of bound ligand (anti-IgG) (Fig. 3A).
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Fluorescence histograms of wt and mutated FcRII isoforms in the mouse B cell line IIA1.6. The relative number of receptors was determined by flow cytometry using the FcRII-specific mAb AT10 (solid line) followed by incubation with FITC-labeled F(ab')2 fragments of goat anti-mouse antibodies. Control cells received the irrelevant mAb MOPC21 (IgG1) (dotted line). The cell number is plotted against the fluorescence intensity on a linear scale (expressed as mean channel value). Clones of IIA1.6 cells transfected with FcRIIb2 wt, FcRIIb1 wt, the respective FcRIIb1 mutants, and FcRIIa-HR used in all further studies.

**Fig. 2.** Fluorescence histograms of wt and mutated FcRII isoforms in the mouse B cell line IIA1.6. The relative number of receptors was determined by flow cytometry using the FcRII-specific mAb AT10 (solid line) followed by incubation with FITC-labeled F(ab')2 fragments of goat anti-mouse antibodies. Control cells received the irrelevant mAb MOPC21 (IgG1) (dotted line). The cell number is plotted against the fluorescence intensity on a linear scale (expressed as mean channel value). Clones of IIA1.6 cells transfected with FcRIIb2 wt, FcRIIb1 wt, the respective FcRIIb1 mutants, and FcRIIa-HR used in all further studies.

**Inhibition of the sIgG-induced Calcium Influx by FcRIIb2 Is Dependent on the Presence of the Carboxyl-terminal Tyrosine Residues**—Cross-linking of the antigen receptor on IIA1.6 cells causes the rapid release of calcium from intracellular stores followed by a prolonged elevated intracellular calcium level (Fig. 7B). Activation of B cells with F(ab')2 fragments of rabbit anti-mouse IgG (RAM-F(ab')2) to cross-link sIgG in the absence of extracellular calcium prevented further calcium signaling. This indicates that the long-lasting enhanced intra-
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Cellular calcium level is due to the opening of plasma membrane calcium channels (Fig. 7A). Both human FcRIib1 (Fig. 7A) and FcRIib2 (Fig. 8A) expressed on mouse B cells efficiently inhibit further calcium signaling by co-cross-linking FcR with sIgG via intact rabbit anti mouse IgG antibodies (RAM). The rapid decay observed in FcRIib1 and FcRIib2 expressing IIA1.6 cells in response to RAM strongly resembles that measured in cells stimulated with F(ab')2 fragments in the absence of extracellular calcium. Deletion of 9 amino acids at the carboxyl terminus of FcRIib2 to 35 cytoplasmic amino acids (FcRIib2-M35, Fig. 8B) had no influence on the inhibition of calcium signaling. Further truncation of the cytoplasmic tail to 25 amino acids (FcRIib2-M25, Fig. 8C) either deleted or altered the region involved in the inhibition of the antigen receptor-induced calcium influx.

Replacement of the carboxyl-terminal tyrosine residues in FcRIib1 (Ty'223) (Fig. 7B) and FcRIib2 (Ty'272) (Fig. 8D) by phenylalanine abolished the FcR-mediated inhibition in calcium signaling. Thus, the same region in the cytoplasmic domain of FcRIib2, but distinct amino acids within this motif, mediate endocytosis of small immune complexes and the down-regulation of the antigen receptor-induced calcium response. Replacement of Ty'258 by a phenylalanine had no influence on the inhibition of the calcium influx (Fig. 7C). Comparison of our results with those obtained by Amigorena et al. (28) for the mouse FcRIib isoforms reveals that a relatively short, conserved stretch of amino acids corresponding to amino acids 26-31 (referred to the cytoplasmic region of human FcRIib2) in the cytoplasmic region of both mouse and human FcRIib isoforms must be involved in the inhibition of calcium signaling. In particular, the presence of the tyrosine residue within the homologous amino acid sequences of the human FcRIib isoforms is essential for the inhibition of the antigen receptor-induced calcium influx.

Co-cross-linking of FcR with sIgG Leads to Tyrosine Phosphorylation of FcRIib1 But Not of FcRIib2 in IIA1.6 Cells—Having demonstrated the functional significance of Ty'223 in FcRIib1 and Ty'272 in FcRIib2 for the inhibition of calcium signaling in B cells we questioned whether these amino acids are targets of protein tyrosine kinases involved in signal transduction via these FcR. FcRIib1 and FcRIib2 expressed in IIA1.6 cells were cross-linked either with RAM-F(ab')2 or the intact rabbit antibody. Stimulation of FcRIib1- or FcRIib2-expressing IIA1.6 cells by sIgG cross-linking for 1, 3, 5, 10, and 15 min did not induce tyrosine phosphorylation of FcRIib1 or FcRIib2. Representative results are shown for FcRIib1- and FcRIib2-expressing

| FcR     | cytoplasmic tail | endocytosis | down-regulation | tyrosine phosphorylation |
|---------|------------------|-------------|-----------------|-------------------------|
| FcRIib2 wt | 247             | 273         |                 |                         |
| FcRIib2 F273 | 247             | F           |                 |                         |
| FcRIib2 M35 | 247             | Y           | 242             |                         |
| FcRIib2 M25 | 247             | 272         |                 |                         |
| FcRIib2 M13 | 247             | 280         |                 |                         |
| FcRIib2 M1  | 247             | 248         |                 |                         |
| FcRIib1 wt  | 247             | 258         | 292             | 310                     |
| FcRIib1 F258 | 247             | F           | Y               | 310                     |
| FcRIib1 F292 | 247             | Y           | F               | 310                     |

Fig. 3. Quantitative measurement of ahIgG uptake via FcR expressed in IIA1.6 cells. A, FcRIib1-HR- (○), FcRIib2 wt- (•), and FcRIib1 wt- (□) expressing cells were incubated with 50 μg/ml ahIgG at 4 °C and then shifted for 2, 5, 15, and 30 min to 37 °C. Remaining ahIgG was measured using F(ab')2 fragments of FITC-labeled goat anti-human IgG antibodies at 4 °C by flow cytometry. The shift in fluorescence intensity was calculated as described under "Materials and Methods." B, endocytosis mediated by FcRIib2 wt- (○), FcRIib2-M35 (□), FcRIib2-M25 (●), FcRIib2-F273 (■), FcRIib1-F258 (□), and FcRIib1-F292 (△). Cells were treated as described in panel A and incubated for 5, 15, and 30 min at 37 °C. The data are mean values from three independent experiments repeated with at least two different cell clones.

Fig. 4. Biological functions mediated by the FcRIib isoforms and receptor mutants. The cytoplasmic tails of the FcRIib isoforms and receptor mutants are schematically presented as bars. The 19 amino acid sequence present in FcRIib1 is highlighted as a bold bar (not drawn to scale). The tyrosine-containing sequence motif of FcRIib1 and FcRIib2 is depicted in the figures by the single amino acid letter code. The positions of tyrosine (Y) residues that have been replaced by phenylalanine are indicated (F) (numbering refers to the start methionine). The designation of the FcRIib2 deletion mutants corresponds to the respective length of the truncated tails. The last amino acid of the transmembrane region is indicated (amino acid 247).
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**Fig. 5.** FcRIIb1 and FcRIIb2 cross-linking on transfected IIA1.6 cells does not stimulate an intracellular calcium signal. Cells preloaded with Fluo-3 and SNARF-1 were assayed by flow cytometry, after stimulation with 35 µg/ml ahIgG (□) at the times indicated by an arrow. A, FcRIIa-HR expressing cells were stimulated with ahIgG either in the presence (□) or absence (△) of extracellular calcium in Krebs-Ringer solution (supplemented with 500 µM EGTA). FcRIIb1 wt (B) and FcRIIb2 wt (C) were stimulated with ahIgG (□); for controls slgG was cross-linked with 20 µg/ml RAM-F(ab')2 (○) known to elicit a rise in the intracellular calcium concentration.

**Fig. 6.** Analysis of FcRIIb tyrosine phosphorylation in signaling and non-signaling mutants. FcRIIb-expressing cells were stimulated at 37 °C with 50 µg/ml of RAM-F(ab')2 fragment for 15 min, with 75 µg/ml RAM for 30 s, and with 25 µg/ml ahIgG for 3 min. A FcRIIb1, b2, and the respective mutants were precipitated with mAb KB61 (+) or an irrelevant slgG1 as control (C). After 10% SDS-polyacrylamide gel electrophoresis and blotting on nitrocellulose filters, phosphorylated proteins were detected with mAb 4G10 and peroxidase-labeled goat anti-mouse antibodies. The phosphorylated FcRIIb1 wt and FcRIIb1-F258 are indicated by an arrow. B, the filters were subsequently reprobed with the anti FcRII mAb IIIA5 and peroxidase-labeled goat anti-mouse IgG antibodies. FcRIIb1, FcRIIb1-F258, and FcRIIb1-F292 are indicated by an arrow, whereas FcRIIb2 wt and FcRIIb2-F273 are indicated by an asterisk.

In contrast to slgG cross-linking, we found that co-cross-linking of FcRIIb1 with the antigen receptor induces tyrosine phosphorylation of FcRIIb1 (Fig. 6A, lane 3). Surprisingly, FcRIIb2 was not phosphorylated under the same conditions (Fig. 6A, lane 9) although, except for the 19 amino acid insertion, its cytoplasmic region is identical with FcRIIb1. This is not the result of different kinetics of FcRIIb2 phosphorylation compared to FcRIIb1 since extensive analyses of the tyrosine phosphorylation at various time points from 30 s to 1 h revealed no tyrosine phosphorylation of FcRIIb2. Due to the insertion, FcRIIb1 contains 1 additional tyrosine residue (Tyr293) compared to FcRIIb2. Replacement of Tyr293 → Phe296 of FcRIIb1 had no influence on the rapidly induced tyrosine phosphorylation in response to FcR-sIgG co-cross-linking with RAM. Since FcRIIb1-F292 was not phosphorylated when co-cross-linked with sIgG (Fig. 6B, lane 8), we conclude that the Tyr292 is the sole tyrosine residue phosphorylated under these conditions. The data presented here are representative for experiments where the FcRIIb1-F292-expressing IIA1.6 cells were stimulated for 30 s to 1 h, and the assays were carried out at least in triplicate. Furthermore, we additionally used rabbit anti-phosphotyrosine antibodies, which also gave negative results of FcRIIb1-Tyr293 and FcRIIb2 tyrosine phosphorylation (results not shown). Therefore, the same tyrosine residue (Tyr293), which is essential for the FcRIIb1wt-mediated down-regulation of the antigen receptor induced calcium signal, becomes phosphorylated under co-cross-linking conditions (Fig. 6A, lane 6). By reprobing the phosphotyrosine blots with the FcRII-specific mAb IIIA5, we verified in each experiment that the molecular mass of the phosphoprotein corresponds to the precipitated FcRIIb1. Although FcRIIb2 and FcRIIb1-F292 were precipitated from the cell lysates in amounts comparable to FcRIIb1 (Fig. 6B), we could not detect tyrosine phosphorylation of FcRIIb2 or FcRIIb1-Tyr293 in Western blots. Thus, although superficially located in the same sequence motif, the tyrosine residues of both FcRIIb isoforms are not equal substrates of protein tyrosine kinases.

Because of the coincidence of FcRIIb1 phosphorylation at position 292 after co-cross-linking with slgG and the loss of function when Tyr292 was changed into an P292, we were interested in whether the kinetics of FcRIIb1 phosphorylation parallels the time course of the decay of the calcium signal. This
might be an indication of a possible involvement of tyrosine phosphorylation of the FcRIIb1 in the processes leading to the regulation of the plasma membrane calcium channel. FcRIIb1 wt-expressing IIA1.6 cells were stimulated with intact rabbit IgG, and FcRIIb mutants expressing IIA1.6 cells were stimulated with intact rabbit IgG. Phosphorylated to precipitated receptor. We observed a very rapid tyrosine phosphorylation of FcRIIb1 after co-cross-linking with anti-IgG, reaching maximum phosphorylation within 30 s after stimulation (Fig. 9A). The phosphorylation of FcRIIb1 did not continue to a plateau level as observed for the antigen receptor-associated Ig-α and Ig-β after sIgG cross-linking (8) but declined to 80% of the initial value within 1 min. After 3 min at 37°C, only 10% of the FcRIIb1 was phosphorylated. Phosphorylation returned to the base line within 5 min. To compare the kinetics of FcRIIb1 phosphorylation with those of the inhibition of the calcium influx, we determined the half-decay time of the calcium response after FcR and sIgG co-cross-linking. This was calculated to be about 30–40 s from the time needed to reach the peak F-fluo-3/SNARF-1 proportion to the half-maximal value and was comparable to that estimated after sIgM cross-linking in calcium-free buffer (Fig. 7A). Thus, the maximum of FcRIIb1 phosphorylation precedes the inhibition of the calcium influx. However, our finding that FcRIIb2 in anti-phosphotyrosine Western blots was not detectably phosphorylated argues against a general role of tyrosine phosphorylation for the FcRIIb1/FcRIIb2-mediated regulation of the calcium channel opening. In addition, we could not observe any significant difference in the half-decay time of the calcium response in FcRIIb1- or FcRIIb2-expressing cells. It is more likely that the phosphorylation of FcRIIb1 is part of a yet uncharacterized signaling cascade leading to the down-regula-
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Figure 9. Kinetics of the FCRIIB1 wt tyrosine phosphorylation after co-cross-linking with sIgG. Cells were stimulated with 75 μg/ml RAM for the times indicated. After immunoprecipitation with the anti-FCRII mAb KB61, the eluted material was subjected to 10% SDS-polyacrylamide gel electrophoresis and electrophoretically blotted on nitrocellulose filters. A, tyrosine-phosphorylated proteins were detected after incubation with anti-phosphotyrosine mAb 4G10 and F(ab')2 fragments of peroxidase-labeled goat anti-mouse lgG antibodies. B, after the removal of bound antibodies blots were subsequently reprobed with the pan-FCRII mAb II1A5 to determine the amount of precipitated receptor. FCRIIB1 is indicated by an arrow in panel C. The relative phosphorylation of FCRIIB1 wt was calculated after densitometric analysis of the phosphotyrosine blot and the FCRIIB1 blot. The ratio of the peak integrals obtained was expressed in percent of the maximum phosphorylation seen after 30 s.

Discussion

Recent studies confirmed the existence of FCRIIB1 and FCRIIB2 transcripts in human mature B cells, whereas mouse B cells only express FCRIIB1 (10, 11). To analyze the signals transduced via the individual isoforms, FCRIIB1 and FCRIIB2 were expressed in the mouse B cell line IIA1.6, which is devoid of the endogenous FCR. However, the expression pattern of key components of the signal transduction cascade, i.e., the protein tyrosine kinases fyn, blk, and yes, is similar in mouse and human B cells (9, 30).

We demonstrated that the human FCRIIB2 in contrast to FCRIIB1 expressed in B cells mediates the endocytosis of small immune complexes, although the amount and time course of the internalization of receptor-bound sIgG are significantly lower compared to the FCRIIA expressed in B cells (11, 13). Endocytosis of immune complexes via the murine FCRIIB2 expressed in IIA1.6 cells has been shown to enhance antigen presentation and the proliferation of an interleukin-2-dependent T cell line (28). Because mouse B cells do not naturally express FCRIIB2 it has been speculated that the mis-regulation of FCRIIB2 expression in B cells may lead to autoimmunity. Therefore, homologous expression of FCRIIB2 in human B cells provides a mechanism leading to the internalization, processing, and presentation of antigen independent of the specificity of the antigen receptor. Previous results have shown that the activation of CD4+ T cells requires a co-stimulatory signal provided by the antigen-presenting cell. In the absence of a co-stimulatory signal, T cells were tolerant to the respective antigen (31, 32). Thus, T cell activation may depend on the state of activation of the antigen-presenting B cell. Moreover, although both FCRIIA and FCRIIB2 mediate the uptake of immune complexes in B cells, FCRIIB2 might be involved in the regulation of cellular processes different from those involving FCRIIA: FCRIIA containing the tyrosine-dependent cell activation motif induces intracellular calcium release in contrast to FCRIIB2 (11, 13, 33). Furthermore, ligand binding to FCRIIB2, in contrast to FCRIIA (13), is not associated with receptor tyrosine phosphorylation. Thus, receptor clustering per se is insufficient to induce FCRIIB phosphorylation.

The sequence motif, which accounts for the coated pit localization of FCRIIB2 recently demonstrated by us (34), can be limited to the last 4 amino acids of AENTITYSLL deleted in the M25 mutant (Fig. 9). Based on these data we suggest that the 2 leucines are essential for the FCRIIB2-mediated endocytosis of small immune complexes.

Soluble FcR generated either by proteolytical cleavage or alternative splicing have been shown to regulate B cell activation (36, 37). Shedding of FCRII has been observed after antigen-driven B cell activation and may involve FCRII phosphorylation (38). Ligation of sIgG on IIA1.6 with RAM-F(ab')2 fragments as a surrogate for antigen did not induce any tyrosine phosphorylation of FCRIIB1 or FCRIIB2. Furthermore, the phosphorylation of mouse FCRIIB1 after antigen receptor cross-linking was on serine and threonine residues (39). Although fyn is associated with FCRII in resting and activated human B cells (30), FCRII was found to be predominantly phosphorylated on serine and threonine residues. Since we could not observe any tyrosine phosphorylation of both FCRIIB isoforms expressed in mouse B cells after antigen receptor cross-linking, we suggest that serine/threonine rather than tyrosine phosphorylation of FCRIIB is probably involved in the shedding of FCRII from B cells.

B cell proliferation can also be inhibited upon sIg-FcR ligation with antigen-antibody complexes (1). Co-cross-linking of either FCRIIB1 or FCRIIB2 with sIg markedly influences the antigen receptor-induced calcium signaling by preventing the sustained, prolonged, elevated calcium level seen after cross-linking sIg with RAM-F(ab')2. In contrast, the first rapid rise in the intracellular calcium concentration due to the opening of inositol trisphosphate-sensitive calcium stores remains unaffected. Thus, engagement of the FCRIIB isoforms interferes with the opening of a yet unknown plasma membrane calcium channel (3). Our data show that the region involved in the inhibition of further calcium signaling overlaps with the region essential for mediating endocytosis since truncation of the cytoplasmic tail to 25 amino acids (FCRIIB2-M25) abolished the negative effect of FCRIIB2 on the calcium influx in B cells. In addition, deletion of the entire region in the mouse FCRIIB2 impaired the negative influence on calcium signaling (28). However, in contrast to the sequence motif required for endocytosis...
cytosis, replacement of the tyrosine residues by phenylalanine affected the receptors' ability to modulate the plasma membrane calcium channel activity. Most notably, although Tyr273 (FcRIIb2) and Tyr289 (FcRIIb1) are located within the same conserved sequence motif they are not equal substrates for protein tyrosine kinases. Surprisingly, only FcRIIb1 and FcRIIb1-F258 were found to become detectably phosphorylated in anti-phosphotyrosine Western blots. Our results clearly demonstrate that FcRIIb1 is phosphorylated on Tyr289, which as has been demonstrated by amino acid sequence alignment is the homologous tyrosine residue to Tyr273 of FcRIIb2. Thus, the 19 amino acid insert in FcRIIb1 not only abolished the endocytosis capacity of FcRIIb1 but positively influenced the accessibility of a possible target sequence for a protein tyrosine kinase yet to be characterized. Moreover, the homologous tyrosine residue in the mouse FcRIIb1 is also essential for the inhibition of the calcium influx and becomes phosphorylated when FcRIIb1 and sIgG are co-cross-linked (40). We suggest, that the inserted 19 amino acid sequence renders the overall tyrosine residue, which only becomes phosphorylated in one receptor isoform (FcRIIb1), makes it difficult to decide whether tyrosine phosphorylation is involved in the FcRIIb-mediated regulation of the plasma membrane calcium channel. Whether the mouse FcRIIb2 isoform in the mouse B cell line IIA1.6 becomes tyrosine phosphorylated when co-cross-linked with sIgG remains to be determined. We suggest that due to the inserted sequence found in FcRIIb1 the human FcRIIb isoforms associate with distinct sets of cytoplasmic effector proteins, which may initiate distinct signal transduction pathways. The characterization of isoform-specific associated cytoplasmic proteins will help to understand the precise role of the tyrosine residues within the cytoplasmic tails of FcRIIb2 and FcRIIb1. Apart from the regulation of calcium signaling in B cells, tyrosine phosphorylation of FcRIIb1 may contribute to the differential regulation of the activity of the two FcRIIb isoforms. Thus, tyrosine phosphorylation could positively or negatively regulate isoform-specific effector functions.

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