Identification of Signaling Motifs Within Human FcγRIIa and FcγRIIb Isoforms

By Ingrid E. Van den Herik-Oudijk, Peter J.A. Capel, Tjomme van der Bruggen, and Jan G.J. Van de Winkel

To assess the functional capacity of the heterogeneous FcγRII (CD32) family and to identify critical regions for functioning, we generated a panel of B-cell transfectants. The FcγR-negative B-cell line IIA1.6 was transfected with wild-type or mutant human FcγRIIa and Iib molecules. Solely FcγRIIa-expressing IIA1.6 cells were capable of phagocytosing opsonized Staphylococcus aureus bacteria, and cross-linking of FcγRIIa triggered a rapid induction of tyrosine phosphorylation after 20 seconds. Analysis of FcγRIIa mutants identified the immunoreceptor tyrosine-based activation motif (ITAM; previously described as ARH-1 motif) within the Ila cytoplasmic tail to be critical for B-cell activation. In contrast, FcγRIIb isoforms triggered tyrosine phosphorylation on cross-linking with much slower kinetics (>3 minutes) than FcγRIIa. Furthermore, solely FcγRIIIb molecules proved capable of downregulating [Ca²⁺], and interleukin-2 production on co-cross-linking with sIgG in IIA1.6. The FcγRIIIb-mediated functions were absent in FcγRIIIb mutants in which the tyrosine or leucine within the YSLL motif is substituted with alanine. In conclusion, these data show the presence of functionally critical motifs within FcγRIIIb cytoplasmic tails. FcγRIIIa contains an ITAM involved in B-cell activating functions, whereas the downregulatory activity of FcγRIIb isoforms is linked to an ITIM.

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MATERIALS AND METHODS

Cells. The murine B-cell line IIA1.6 was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 µg/mL gentamycin, HEPES, 1 g/L NaHCO₃, and 2 mM/L sodium pyruvate. IIA1.6 cells expressing various human FcγRII cDNAs were cultured in the presence of genetin (G418, 0.8 mg/mL; Gibco BRL, Grand Island, NY) and were obtained as described. In short, the pRC-CMV vector containing the coding sequence for human FcγRIIb (pIP9), wild-type (wt) FcγRIIb (pIP14), mutant FcγRIIb* (mutant FcγRIIa, or FcγRIIb2 (pIP4) cDNAs as inserts were linearized with Sca I. FcγRIIb* mutants, Iib1*Y45F and Iib1*L48F, represent molecules in which the tyrosine at aa position 45 or the leucine at aa position 48 in the cytoplasmic tail were

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Submitted September 13, 1994; accepted November 22, 1994.

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0006-4971/95/8508-0008$3.00/0

mutated into phenylalanine (Fig 1). FcγRIIa mutants, IlaY48F, IIaY64F, and IlaY48F-Y64F, represent molecules in which one or both tyrosines of the ITAM motif were mutated into a phenylalanine (Fig 1). All mutant receptors were generated by a two-step overlap extension polymerase chain reaction (PCR) method. Asp23-57, seek for information.

**Phagocytosis assay.** *Staphylococcus aureus* Wood (a variant deficient in protein A expression) bacteria were cultured overnight at 37°C in Muller Hinton Broth, harvested by centrifugation at 2,500 rpm for 10 minutes, washed three times with phosphate-buffered saline (PBS), and labeled by incubation with fluorescein isothiocyanate (FITC; Sigma, St Louis, MO) at a concentration of 0.4 mg/mL in 0.1 mol/L NaH₂PO₄/Na₂HPO₄ buffer, pH 9.6, for 30 minutes at 4°C. After three washes with Hank’s buffered salt solution (HBSS), pH 7.3, 1 x 10⁵ bacteria/mL were opsonized with HBSS containing 15% (vol/vol) heat-inactivated pooled human serum by incubation for 30 minutes at 37°C. After washing, opsonized FITC-labeled bacteria were incubated with FcγRII-expressing IIA1.6 B cells in RPMI 1640 medium (containing 10% FCS) for 45 minutes at 4°C. After two washes, cells were further incubated for 45 minutes either at 4°C or 37°C. Remaining cell-surface-bound bacteria were detected by incubation for 30 minutes at 4°C with either RTIC-conjugated goat antihuman IgGκ/λ antisera (Southern Biotechnology, Birmingham, AL) for microscopic analyses or phycoerythrin (PE)-conjugated goat antihuman IgGκ/λ antisera (Southern Biotechnology) for flow cytometric quantitation of 5,000 cells in each sample. FITC and PE fluorescence intensities of cells maintained at 4°C throughout served as control for binding of bacteria (0% phagocytosis), whereas the decrease of PE fluorescence intensity on

**Fig 1.** Alignment of cytoplasmic regions of FcγRII isoforms and variants used in this study. Amino acid structures of the different FcγRIIα-cytoplasmic tails are shown relative to the human FcγRIIα isoform. A conserved (ITM) motif with mouse FcγRIIb is indicated for comparison. Deletions are represented by dashed lines, with the number of deleted amino acids in brackets. Stretches with identical aa are indicated by solid lines. wt or mutated YXXL motifs are underlined and the FcγRα-ITAM motif is boxed. Mutations within ITAM/ITIM motifs, the tyrosine (Y) at position 11 of human FcγRIIb and aspartic acid (D) within FcγRIIb* at this position are presented in bold. Stop codons are marked by asterisks.

**Fig 2.** FcγRII expression levels in murine IIA1.6 B-cell transfec-
tants. Cells were incubated with MoAb AT10 () or immuno-
fluorescence buffer alone (shaded area) followed by FITC-labeled goat antihuman IgGκ/λ antisera. Fluorescence was recorded as arbitrary units on a logarithmic scale and plotted against the relative cell number. Panels represent IIA1.6 cells transfected with FcγRIIα (a), IIaY64F (b), IIaY48F (c), IIa (d), IIaY64F (e), Ila (f), IlaY48F (g), IlaY48F-Y64F (h), IlaY64F (i), and Ila tail (j).

**Table:**

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incubation at 37°C reflected bacterial phagocytosis. The dot-plot
diagrams of FITC and PE fluorescence intensities served to quanti-
tate the percentage of cells with decreased PE fluorescence by using
electronic gating. Cells located on the diagonal in dot-plot diagrams
bear externally bound bacteria (stained both with FITC and PE la-
beis). Electronic gates were set below this diagonal (see Fig 4A) to
quantitate cells showing a decrease in PE fluorescence levels on
incubation at 37°C (because of bacterial phagocytosis).

The role of tyrosine phosphorylation in bacterial phagocytosis was assessed by using herbycinic A20 and genistein.26 Cells were pretreated with 500 ng/mL genistein (Sigma) for 30 minutes or with 2 ng/mL herbycinic A (GIBCO Laboratories, Grand Island, NY) for 18 hours before the phagocytosis assay. The involvement of the cytoskeleton in phagocytosis was studied on preincubation with 200 ng/mL cytochalasin D (Sigma) for 30 minutes at 37°C. Incubation of cells in RPMI 1640 medium with 0.2% dimethyl sulfoxide (DMSO; Solvent for genistein, herbycinic A, and cytochalasin D) served as control. Inhibitors were present throughout the experiments.

The addition of nonopsonized S aureus to FcγRII-expressing cells and of opsonized S aureus to untransfected IIA1.6 cells served as specificity controls in phagocytosis assays.

Tyrosine phosphorylation assays. IIA1.6 B cells expressing different FcγRII isoforms were incubated with CD32 monoclonal antibody (MoAb AT10 culture supernatant (mIgG1; Serotec, Bicester, UK) or KB61 ascites (mIgG1) for 30 minutes at room temperature, washed twice with RPMI 1640 medium, and suspended in different tubes in RPMI 1640 medium (2 × 10^5 cells/50 μL). Goat antoismatch IgG1 antiserum (Southern Biotechnology; final concentration, 20 μg/mL) was added for different time periods at 37°C to cross-link FcγRII. Reactions were stopped by the addition of 70 μL reducing solvent for genistein, herbibycin A, and cytochalasin D) served as
specificity controls in phagocytosis assays.

Tyrosine phosphorylation assays. IIA1.6 B cells expressing different FcγRII isoforms were incubated with CD32 monoclonal antibody (MoAb AT10 culture supernatant (mIgG1; Serotec, Bicester, UK) or KB61 ascites (mIgG1) for 30 minutes at room temperature,

To study IL-2 production triggered by cross-linking FcγRII, IIA1.6 cells expressing various FcγRII molecules were incubated with CD32 MoAb AT10 culture supernatant for 30 minutes at room temperature. After washing, goat antoismatch IgG1 antiserum (Southern Biotechnology; final concentration, 30 μg/mL) was added for 24 hours at 37°C. Alternatively, heat-aggregated human IgG2 was coated onto 96-well plates or was added to cell suspensions (concentration, 1 to 1,000 μg/mL).

The presence of IL-2 released by the IIA1.6 cells into culture supernatants was determined by culturing 1 × 10^6 CRTL-2 IL-2-dependent cells with the various culture supernatants in 96-well plates. After 24 hours, 1 μCi of 3H-thymidine was added to each well and cells were harvested 4 hours later onto glass fiber filters (Wallac, Turku, Finland) for liquid scintillation counting.

Calcium mobilization assays. The effect of co-cross-linking slgG with FcγRIIb1* and FcγRIla mutants expressed on IIA1.6 B cells on calcium mobilization was performed exactly as described.20 Briefly, Fluor-3/ SNARF-1–loaded cells were run on a flow cytometer for 24 seconds, followed by the addition of either F(ab')2 fragments of goat antoismatch IgG (Southern Biotechnology; final concentration, 5 μg/mL) or intact goat antoismatch IgG (Southern Biotechnology; final concentration, 7.5 μg/mL). To evaluate the capacity of the various FcγRII mutants to directly trigger an increase in [Ca²⁺], Fluor-3/SNARF-1–loaded cells were incubated with MoAb AT10 for 20 minutes at room temperature (in the dark). Cells were washed and run on a flow cytometer for 24 seconds and goat antoismatch IgG1 Ab (Southern Biotechnology; final concentration, 6 μg/mL) was added to cross-link FcγR. Alternatively, cross-linking of FcγR was performed by addition of heat-aggregated human IgG (final concentration, 30 μg/mL) at 24 seconds.

RESULTS

To identify the cytoplasmic regions critical for human FcγRIla and Iib functioning, we generated a panel of stable wt and mutant transfectants in the FcR-negative and slgG2a-positive mouse B-cell line IIA1.6, which is derived from the A20/21 lymphoma cell line.22 We stably transfected wt FcγRIIb1, Iib2, Iib1* (a variant Iib1 cDNA bearing an aspartic acid instead of tyrosine at aa position 11 in the cytoplasmic tail), and Iia.20 Also, an FcγRIIb variant (Iib23Δ57) cDNA, found on screening a Raji cDNA library and bearing a deletion in its cytoplasmic tail of amino acids 23 to 57 (Fig 1), was transfected. In addition, we transfected two Iib1* mutant receptors in which either the tyrosine at a position 45 (Iib1*Y45F) or the leucine at aa position 48 (Iib1*L48F) within the cytoplasmic tails were mutated into phenylalanines. Furthermore, we generated FcγRIla mutants in which one (IiaY48F and IiaY64F) or both tyrosines (IiaY48F-Y64F) within the ITAM present in FcγRIla cytoplasmic tail were changed into phenylalanines. Expression levels of the different transfectants were comparable and remained stable during the course of experiments (Fig 2).

Phagocytosis of S aureus by IIA1.6 B lymphocytes expressing FcγRII molecules. To study the phagocytic capacity of IIA1.6 B cells expressing various FcγRII molecules, a new method was developed by using IgG opsonized FITC-labeled S aureus bacteria. On incubation at 4°C to allow binding of opsonized bacteria, temperature was shifted to 37°C (enabling phagocytosis), followed by determination of bacteria remaining at the cell surface via incubation with FITC-labeled goat antianti-IgG antiserum. Microscopic analysis of IIA1.6 cells expressing FcγRIIb1* and Iia is shown in Fig 3, and different fluorescence filters were used to detect either the total amount of bound/phagocytosed FITC-labeled bacteria (Fig 3B and E) or the nonphagocytosed FITC-labeled bacteria (Fig 3C and F). FcγRIla-expressing cells showed efficient binding and phagocytosis of S aureus, whereas Iib1* was able to bind but not to ingest bacteria. We.
Phase - Contrast  Internal + External  External

Phase - Contrast  Internal + External  External

Furthermore, used this new method to analyze the phagocytic capacity of IIA1.6 B cells by flow cytometry. FITC fluorescence served to detect the total amount of bound/phagocytosed bacteria and PE-labeled goat antihuman IgG antiserum served to assay nonphagocytosed S aureus. Dot-plot diagrams of FITC and PE fluorescence intensities of FcγRIIb2, Ila, IlaY48F-Y64F, and Ila-tail-expressing IIA1.6 cells either incubated at 4°C or 37°C, are shown in Fig 4A. Cells expressing FcγRIIb1, wt, and Iib1* mutants, Iib2, Iib3Δ57 and Ila-tail− showed comparable PE-fluorescence intensities on incubation at 4°C and 37°C, indicating that no bacteria were phagocytosed (Fig 4B). The decrease in PE fluorescence intensities observed in Ila-expressing cells on incubation at 37°C suggested this subclass to be the sole FcγRII isoform capable of directing phagocytosis by IIA1.6 cells. A phagocytic process was further supported by the decrease in FITC fluorescence intensity (Fig 4A, panel d), which is consistent with quenching caused by uptake of FITC-labeled bacteria into low-pH environments. Setting of electronic gates within dot-plot diagrams was used for semiquantitative assessment of the percentage of cells that phagocytosed S aureus (see Materials and Methods). These analyses showed about 65% of the FcγRIIa-expressing cells (5 individual experiments) that had bound bacteria to be located within this gate, suggesting a rather efficient phagocytic capacity. To evaluate whether the two tyrosines within the ITAM (Fig 1) are critical for phagocytosis in B cells, we tested mutant Ila-expressing cells. All three FcγRIIa mutant-expressing cell lines exhibited a strongly reduced phagocytic capacity (>80%; see Materials and Methods). These analyses showed about 65% of the FcγRIIa-expressing cells (5 individual experiments) that had bound bacteria to be located within this gate, suggesting a rather efficient phagocytic capacity. Consistent with this observation, no detectable level of phagocytosis was found for FcγRIIa-tail− expressing cells (Fig 4B).

To study involvement of the cytoskeleton in phagocytosis, IIA1.6 cells were preincubated with 200 ng/mL cytochalasin...
Fig 4. Flow cytomtric quantitation of phagocytic capacity of IIa1.6 B cells. FITC-labeled IgG-opsonized S aureus were incubated with IIa1.6 cells expressing different FcyRII isoforms for 45 minutes at 4°C. Cells were then incubated either at 4°C or 37°C for 45 minutes and the remaining surface-bound S aureus were detected by the addition of PE-conjugated goat antihuman antiserum. FITC and PE fluorescence of 5,000 cells was quantitated by flow cytometry and dot-plot diagrams are shown in (A). Mean PE-fluorescence intensities of cells incubated either at 4°C (III) or 37°C (III) are shown in (B). The experiment was repeated at least five times with essentially identical results.

D for 30 minutes at 37°C. Ingestion of bacteria by FcyRIIa-expressing cells was found to be blocked completely, indicating a critical role for microfilament organization in B-cell uptake of bacteria (data not shown; n = 3).

The capacity of the IIa mutants to ingest complexes consisting of CD32 MoAb AT10 and goat antimouse IgG1 was assayed as described in Van den Herik-Oudijk et al.20 FcyRIIa was found to internalize 80% ± 5% of the FcyRII-MoAb complexes within 5 minutes (n = 3; also described in Van den Herik-Oudijk et al.20). Interestingly, FcyRIIaY48F internalized 73% ± 5% of the complexes (n = 3), IlbY64F 75% ± 7%, and IIaY48F-Y64F 67% ± 5% (n = 3) within 5 minutes. These data show the tyrosines within the ITAM not to be essential for internalization of FcyRII-MoAb complexes via FcyRIIa.

Sensitivity to tyrosine kinase inhibitors suggested an important role for tyrosine phosphorylation in phagocytosis by professional phagocytes.21 Therefore, we assessed the involvement of tyrosine kinases in phagocytosis by FcyRIIa-expressing IIa1.6 B cells, using the inhibitors genistein and herbimycin A. A total of 500 ng/mL of genistein or 2 ng/mL herbimycin A blocked phagocytosis of opsonized S aureus for ≈80% (3 individual experiments; data not shown). This finding suggested tyrosine phosphorylation to be involved in phagocytosis by IIa1.6 B lymphocytes. Viability of the cells on treatment with genistein and herbimycin A was checked by trypan blue dye exclusion and was always greater than 95%. Treatment of cells with 0.2% DMSO (the solvent for genistein and herbimycin A) had no effect on either binding or uptake of bacteria.

Aggregation of FcyRII isoforms triggers complex tyrosine phosphorylation patterns in B lymphocytes. Cross-linking of surface IgG receptors on B cells results in complex patterns of tyrosine phosphorylated proteins.32-34 Cross-linking of slgG2a expressed on IIa1.6 mouse B cells, indeed, triggered an induction of tyrosine phosphorylation as early as 20 seconds, increasing to a maximum at 2 minutes. Expression of FcyRIIb1, IIb1* wt and mutants, IIb2, IIb23A57, IIa wt, and mutants on IIa1.6 cells had no effect on the tyrosine phosphorylation pattern on cross-linking slgG2a with F(ab')2 fragments of goat antimouse IgG antiserum (Fig 5).

We next studied induction of tyrosine phosphorylation on cross-linking the different FcyRII molecules themselves. FcyRII-expressing IIa1.6 cells were incubated with CD32 MoAb AT10 for 30 minutes at room temperature. After washing, FcyRII-AT10 complexes were cross-linked by incubation with a goat antimouse IgG1-specific antibody for different time periods. Tyrosine phosphorylation in response to cross-linking of FcyRII was detectable as early as 20 seconds and decreased after ≈2 minutes (Fig 5D; summarized in Table 1). This rapid kinetic was comparable to that found on slgG2a cross-linking; in addition, the pattern of tyrosine phosphorylated proteins was very similar. The role of the ITAM in IIa-mediated signal transduction was evaluated by cross-linking of IIaY48F, IIaY64F, and IIaY48F-Y64F FcyR. None of these three mutants was able to increase tyrosine phosphorylation above background levels, showing both tyrosines to be critical for induction of phosphoproteins in IIa1.6 cells (Fig 5E and F). The specificity
of the tyrosine phosphorylation patterns was evaluated on the addition of goat antimes I gG1 to FcγRII-expressing cells that were not preincubated with CD32 MoAb AT10 or KB61. No detectable tyrosine phosphorylation was observed, indicating that the goat antimes I gG1 antiserum did not cross-react with sIgG2a receptors on the IIA1.6 cell membrane (data not shown). In addition, another activation function of FcγRIIa in B cells involves an increase in [Ca²⁺], on cross-linking which was also tested for the various FcγRIIa mutants. However, none of the three IIa mutants initiates an increase in [Ca²⁺], in contrast to FcγRIIa, in two independent experiments.

We next assessed the capacity of the different FcγRIIb molecules to trigger tyrosine phosphorylation in IIA1.6 cells on cross-linking. Remarkably, IIA1.6 cells expressing FcγRIIbl, IIb1*, and IIb2 showed a comparable pattern of phosphoproteins on FcγRII cross-linking that was detectable 3 minutes after stimulation and persisted for 60 minutes triggered either via cross-linking AT10 or KB61 CD32 MoAb (Fig 5A and data not shown). This finding clearly indicated these FcγRIIb molecules to be coupled to signal transducing pathways involving tyrosine phosphorylation in IIA1.6 cells, albeit that the kinetics of phosphorylation were slow. The overall pattern of tyrosine phosphorylated proteins detected on cross-linking FcγRIIa, sIgG2a, or FcγRIIb molecules were found to be rather similar (Fig 5).

Comparison between human and mouse FcγRIIb molecules pointed to a conserved 13 aa region in which a YSLL (Fig 5B and C). Consistent with this, the FcγRIIb23A57 variant molecule was neither capable of increasing tyrosine phosphorylation levels on cross-linking (data not shown).

The effect of co–cross-linking FcγRII and sIgG2a expressed on IIA1.6 cells on tyrosine kinase activity in FcγRIIiexpressing IIA1.6 cells was tested by the addition of whole IgG or F(ab'); fragments of goat antimes I gG antiserum.

No differences in phosphorylation kinetics were observed for any of the FcγRII wt or mutant transfectants. This finding showed co–cross-linking of FcγRII with sIgG not to result in altered tyrosine kinase activities in IIA1.6 cells (data not shown; n = 2).

The ITIM within FcγRIIb is critical for downregulation of [Ca²⁺], increases and IL-2 production on co–cross-linking IIA1.6 sIgG2a and FcγRII molecules. FcγRIIb molecules have previously been shown capable of downregulating [Ca²⁺], on co–cross-linking with sIgG. To evaluate whether the ITIM is involved in this function, we tested the FcγRIIbl*Y45F and IIbl*L48F mutants. Both mutants were unable to affect [Ca²⁺], on co–cross-linking with sIgG, indicating a critical role for both these aa in this early B-cell activation event (n = 3; data not shown).

IIA1.6 cells have previously been shown capable of IL-2 production on activation via sIgG2a. To assess the capacity of human FcγRII-expressing IIA1.6 cells to trigger IL-2 production as a late event of cell activation, cells were incubated overnight in 96-well plates with F(ab'); fragments of goat antimes I gG1 antiserum. All wt and mutant FcγRII-expressing cells were found capable of producing IL-2, as determined by incubation of the IIA1.6 cell supernatants with an IL-2-dependent cell line (CTLL; Fig 6).

We then studied the effect of co–cross-linking FcγRII molecules and sIgG2a on IL-2 production. FcγRIIb-transfected IIA1.6 cells were incubated overnight with whole goat antimes I gG. Notably, FcγRIIbl, IIb1*, and IIb2 molecules downregulated IL-2 production on co–cross-linking with sIgG, whereas the FcγRIIbl23A57, IIbl*Y45F, and IIbl*L48F molecules proved inactive in this respect (Fig 6A). This, again, pointed at a critical functional role for both the tyrosine and leucine within the FcγRIIb ITIM motif. Moreover, wt or mutant FcγRIIa were unable to downregulate IL-2 production (Fig 6B).

To assess whether wt and mutant FcγRIIbl and IIa molecules were themselves able to trigger an increase in IL-2 production on cross-linking, cells were incubated with CD32 MoAb AT10. This was followed by cross-linking of FcγRIIAT10 complexes by the addition of goat antimes I gG1-specific antiserum for 24 hours at 37°C. In addition, aggregation of FcγRII molecules via the ‘‘natural ligand’’ for these low-affinity receptors was performed by incubation of
Fig 5. Induction of tyrosine phosphorylation on cross-linking FcγRIIb1* (a), llb1*Y45F (b), llb1*L48F (c), lla (d), llaY48F (e), and llaY64F (f) molecules expressed in IIa1.6 B cells. IIa1.6 cells transfected with the various FcγRII molecules were incubated with CD32 MoAb AT10 for 30 minutes at room temperature and were washed twice with RPMI 1640 medium. Cells were then incubated with goat antimouse IgG1 antiserum for the indicated time periods, followed by immediate lysis of cells by addition of sample buffer. Samples were loaded onto 7.5% SDS-PAGE gels, blotted to nitrocellulose, and analyzed with an antiphosphotyrosine MoAb. Experiments were repeated at least four times, and representative data are shown. Molecular weights of marker proteins are indicated on the left.
an ITAM (previously indicated as ARH-1) present in the cytoplasmic tail of different signaling subunits (such as FcR γ- and ζ-chains) and a similar, albeit nonidentical, ITAM in FcyRIla. An important role for the FcyRIla ITAM in bacterial uptake was clearly shown by FcyRIla mutants in which the tyrosines were converted into phenylalanines (Fig 4B; Table 1). A role for tyrosine phosphorylation in FcyR-mediated phagocytosis has been reported for professional phagocytes, as illustrated by the sensitivity of this process to genistein and herbimycin A.31 The activation pathway associated with FcyRIla-mediated phagocytosis in B cells may involve similar cellular components because of comparable sensitivity to tyrosine phosphorylation inhibitors (data not shown).

FcyRIla was previously found capable of triggering an increase in [Ca²⁺], on cross-linking; in addition, we describe here a rapid type of tyrosine phosphorylation (Fig 5D). Again, a crucial role for the FcyRIla ITAM was supported by inability of the FcyRIla tyrosine mutants to trigger tyrosine phosphorylation (Fig 5E and F) or an increase in [Ca²⁺], (data not shown; Table 1). However, this motif was clearly not required for internalization of FcyR-MoAb complexes, as shown by the rapid uptake in all FcyRIla mutants (Table 1). In addition, the difference between bacterial uptake and internalization of FcyR-MoAb complexes observed for the Ila mutants clearly suggests a fundamentally distinct mechanism to underly these two processes. The critical role of the FcyRIla ITAM in phagocytosis, ie, triggering of tyrosine phosphorylation and increase in [Ca²⁺], but not internalization, has also been observed in P388.D1 cells.35 The importance of ITAM in FcyRIla signal transduction is, furthermore, supported by recent findings that a phosphorylated Y-x-x-L/I sequence forms the predicted binding site for several members of the Src family protein tyrosine kinase-SH2 domains.36 The physiologic implications of activatory FcyRIla molecules in B cells are unclear. Interestingly, however, early B cells were shown to express Ila as the sole FcγRII molecule present.30 It does not seem too far-fetched, therefore, to postulate a role for FcyRIla in growth/development of early B cells, as recently suggested for FcyRII in T-lymphocyte development.39

Studies with mouse FcyRIII (which is considered the homologue of human FcyRIIa40) showed association with FcR γ-chain to be essential for receptor signaling.6,41,42 Interestingly, cross-linking of mFcyRIII γ-chain complexes in Ila.6-transfected cells triggered an increase in IL-2 production,24 which we did not find for human FcyRIla. This difference between the two receptors may either be caused by higher expression levels of the mFcyRIII γ-chain complexes or by the higher number of ITAM in murine FcyRIII receptor complexes. This last possibility is supported by studies of Irving et al,43 which showed a quantitative relationship between the number of ITAM and the capacity to trigger functions in T cells.43

A hallmark in B-lymphocyte biology is the capacity of FcyR to serve as downregulatory molecules.44,45 All three human FcyRIIb isotypes were previously found to downregulate an increase in [Ca²⁺], on co-cross-linking FcyR molecules with surface IgG2a in IIA.6 B cells.29 Studies with the homologous mouse FcyRIIib1 and mIIb2 isofoms yielded
In this report, the downregulatory effect of FcγRIIB receptors was studied with respect to IL-2 production as a prototypic late event in cell activation. Human FcγRIIb1, IIb1*, and IIb2 all proved capable of inhibiting the production of IL-2 on co-cross-linking with the sIgG receptor on IIA1.6 B cells (Fig 6A and Table 1). Human FcγRIIb isoforms, furthermore, were able to trigger an increased tyrosine phosphorylation pattern in B cells (Fig 5 and Table 1). A comparable pattern of tyrosine phosphorylated proteins was found on cross-linking FcγRIIa or sIgG receptors, albeit that activation kinetics of FcγRIIb and these last two receptors were profoundly different. Cross-linking of FcγRIIa triggered a rapid tyrosine phosphorylation, whereas IIb1, IIb1*, and IIb2 showed a much slower response (Fig 5A and data not shown). This may point at distinct, but overlapping, signaling pathways used by the FcγRIIa and IIb molecules. Notably, murine FcγRIIb proved inactive in triggering enhanced tyrosine phosphorylation upon cross-linking.34 In our view, this apparent discrepancy might be attributable to methodologic differences (mouse FcγRIIb molecules were cross-linked for only 10 minutes; also, a different protocol was used, i.e., a directly conjugated antiphosphotyrosine MoAb).45

We recently obtained a variant cDNA, FcγRIIb23Δ57, in which aa 23 to 57 were deleted relative to IIb1 (Fig 1). On transfection to IIA1.6 B cells, this mutant proved inactive in triggering capping, internalization, bacterial phagocytosis, or tyrosine phosphorylation signals after cross-linking. More interestingly, the inhibitory function associated with all three FcγRIIb molecules (eg, downregulation of IL-2 production [Fig 6A] and [Ca2+]i) on co-cross-linking with sIgG was absent in this mutant. This finding suggested that the region between aa 23 and 57 within the cytoplasmic tail was critical for FcγRIIb-mediated functions. On comparing the aa sequences of the different FcγRII molecules, we noted a 13-aa region in the cytoplasmic tail present in all human FcγRIIb isoforms but absent in FcγRIIa, which was highly homologous to mouse FcγRIIb (see Fig 1). Furthermore, a homology search revealed this 13 aa region to be conserved in rat, 46 guinea pig, 47 and cattle (EMBL databank) FcγRIIb molecules. Within this 13-aa stretch, an ITIM is located. To study the role of the tyrosine and leucine within this motif, FcγRIIb1*Y45F and IIb1*L48F were generated in which each of the aa were mutated into phenylalanines. The critical role for these aa was supported by (1) the absence of effects on [Ca2+]i (data not shown) and IL-2 production (Fig 6A) on co-cross-linking with sIgG and (2) the lack of triggering tyrosine phosphorylation signals on cross-linking (Fig 5 and Table 1). Moreover, deletion studies performed with mouse FcγRIIb also showed this 13 aa region to be essential for functioning of murine FcγRIIb. 46,49 The role of the ITIM within mouse FcγRIIb is also supported by mutation studies. Both tyrosine and leucine at aa positions 73 and 76, respectively, of mFcγRIIb1 were found critical for inhibition of sIgG-stimulated calcium influx and IL-2 production in murine B lymphocytes. 46,50

In conclusion, FcγRIIa and IIb functioning in B cells is regulated via different motifs located in their cytoplasmic tails. The FcγRIIa ITAM motif is critical for activation functions such as phagocytosis and triggering of rapid tyrosine phosphorylation. By contrast, human FcγRIIb molecules were found capable of downregulating B lymphocytes triggered via their surface IgG receptors. Furthermore, these FcγRIIb molecules proved capable of initiating tyrosine phosphorylation patterns. For these FcγRIIb activities, both the tyrosine and leucine within the ITIM are critical. A detailed understanding of the interaction of the conserved activation motifs within FcγRIIa and IIb cytoplasmic tails with cellular components is crucial to appreciate the biologic role of FcγR in B-cell functioning.

ACKNOWLEDGMENT

The authors thank Ingmar Heijnen for expert advice on the phagocytosis assay and Drs Hans Clevers, Sjie Verbeek, and Craig Morton for critical reading of the manuscript.

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Identification of signaling motifs within human Fc gamma RIIa and Fc gamma RIIb isoforms

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