The inhibitory potential of Fc receptor homolog 4 on memory B cells

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Fc receptor homolog 4 (FcRH4) is a B cell-specific member of the recently identified family of FcRHs whose intracellular domain contains three potential immunoreceptor tyrosine-based inhibitory motifs (ITIMs). The signaling potential of this receptor, shown here to be preferentially expressed by memory B cells, was compared with the inhibitory receptor FcγRIIb in B cells expressing either WT FcγRIIb or chimeric proteins in which the intracellular domain of FcRH4 was fused to the transmembrane and extracellular domains of FcγRIIb. Coligation of the FcγRIIb/FcRH4 chimeric protein with the B cell receptor (BCR) led to tyrosine phosphorylation of the two membrane-distal tyrosines and profound inhibition of BCR-mediated calcium mobilization, whole cell tyrosine phosphorylation, and mitogen-activated protein (MAP)-kinase activation. Mutational analysis of the FcRH4 cytoplasmic region indicated that the two membrane-distal ITIMs are essential for this inhibitory potential. Phosphopeptides corresponding to these ITIMs could bind the Src homology 2 (SH2) domain-containing tyrosine phosphatases SHP-1 and SHP-2, which associated with the WT FcRH4 and with mutants having inhibitory capability. These findings indicate the potential for FcRH4 to abort B cell receptor signaling by recruiting SHP-1 and SHP-2 to its two membrane distal ITIMs.

B cell receptor (BCR) engagement initiates a variety of signal transduction pathways, including those leading to the activation of phospholipase C γ (PLCγ), Ras, and phosphatidylinositol 3-kinase. The physiological consequences range from cellular proliferation and differentiation to apoptotic cell death depending on the differentiation status of the B cell, the strength of activating signals, and the presence or absence of costimulatory or inhibitory signals (1). An important mechanism for down-regulating BCR signaling involves its coligation with FcγRIIb, a low affinity receptor for soluble IgG antibody–antigen complexes (2). This signal dampening occurs through phosphorylation of the tyrosine in the immunoreceptor tyrosine-based inhibitory motif (ITIM) of FcγRIIb that is mediated by BCR recruitment of src family kinases. The phosphorylated ITIM is recognized by the Src homology 2 (SH2) domain of the inositol phosphatase SHIP (3–5), which then dephosphorylates phosphatidyl-inositol (3, 4, 5) tris-phosphate (PIP3) to generate PI(3,4)P2 and inorganic phosphate (6). This decrease in PIP3 results in reduced activity of PH domain-containing proteins such as Btk and PLCγ (7). In addition, phosphorylation of SHIP on its NPxY motifs provides docking sites for phosphotyrosine-binding (PTB) domain-containing proteins. One such protein, the p62 Dok adaptor protein, becomes heavily tyrosine-phosphorylated on BCR crosslinking and in turn recruits p120 RasGAP to FcγRIIb (8–10). The combined influence of the effector proteins engaged by FcγRIIb results in an inhibition of BCR signaling that is reflected by reductions in calcium mobilization, mitogen-activated protein kinase activation, and phosphatidylinositol 3-kinase signaling. The function of FcγRIIb is thus mediated primarily via SHIP whereas other inhibitory receptors such as killer cell Ig-like receptor (KIR) and PD-1 recruit the cytosolic tyrosine phosphatases SHP-1 (SH2 domain-containing tyrosine phosphatase 1) and SHP-2, respectively, to modulate signaling cascades (11). Impairment of the mechanisms that negatively regulate BCR signaling is implicated in the pathogenesis of B cell hyperactivity and autoimmunity (12, 13).

Fc receptor homolog 4 (FcRH4) is a member of a recently identified family of Ig domain-containing cell surface receptors that are preferentially expressed on B cells. The Fc receptor homologs that we identified on the basis of their similarity to the classical Fc receptors (14) were also identified as Ig superfamily receptor translocation-associated genes (15); hence, they are also called IRTAs. FcRH4 (IRTA1) is a candidate inhibitory receptor because its intracellular domain contains three tyrosine residues in the midst of amino acid sequences that match the ITIM consensus S/L/V/IxYxxL/V (16–18). To characterize the functional potential of the ITIMs in FcRH4, we have generated chimeric constructs, encoding fusion proteins that consist of the extracellular and transmembrane domains of FcγRIIb and the intracellular domain of FcRH4. For comparison, full-length FcγRIIb constructs were also used in these studies. The chimeric constructs were used to determine that the FcRH4 intracellular domain exerts a potent negative regulatory effect on BCR signaling by inhibiting whole cell tyrosine phosphorylation, mitogen-activated protein kinase activation, Akt-activation, and induction of calcium mobilization. This analysis indicates that the inhibitory function of the intracellular domain of FcRH4, in contrast to FcγRIIb, is mediated by the recruitment of SHP-1 and SHP-2 to the two membrane-distal ITIMs. The further demonstration that FcRH4 transcripts are preferentially expressed in memory B cells implies an important regulatory potential for this inhibitory receptor in modulating secondary antibody responses.

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Materials and Methods

Cells and Antibodies. A20-IIA1.6 B cells were maintained in RPMI medium 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, and 50 μM 2-mercaptoethanol. BOSC23 cells were grown in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin. Anti-hemagglutinin (HA)-antibody 12CA5 was obtained from Roche Diagnostics (Mannheim, Germany), the anti-mitogen-activated protein kinase antibodies, and anti-Akt antibodies from Cell Signaling Technologies (Beverly, MA), horseradish peroxidase-coupled anti-phosphotyrosine antibodies 4G10 from Upstate Biotechnologies (Lake Placid, NY), anti-PLCγ2, anti-SHP-1, anti-SHP-2, and anti-SHIP antibodies

Abbreviations: BCR, B cell receptor; PLC, phospholipase C; ITIM, immunoreceptor tyrosine-based inhibitory motif; SH2, Src homology 2; SHIP, SH2-containing inositol phosphatase; SHP, SH2 domain-containing tyrosine phosphatase; FcRH4, Fc receptor homolog 4; HA, hemagglutinin.

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from Santa Cruz Biotechnology and the whole IgG and F(ab′)$_2$ fragments against murine Ig from Zymed.

Generation of Chimeric Mutants of FcRH4. Site-directed mutagenesis was performed by PCR according to standard protocols. The mutated cDNAs encoding the intracellular domain of FcRH4 were subcloned into pBluescript and verified by DNA sequencing. These were fused to cDNAs encoding the extracellular and transmembrane domains of murine FcγRIIb and cloned into pDisplay (Invitrogen). A *Not*I site was introduced between the transmembrane domain of FcγRIIb and the intracellular domain of FcRH4, which translates into a 3-alanine spacer. For retroviral transduction, cDNAs encoding the chimeric proteins were excised from pDisplay and cloned into pMX-PIE, a retroviral expression vector, which expresses the gene of interest upstream of an internal ribosomal entry site (IRES) element and the enhanced green fluorescent protein (a gift from A. Mui, Jack Bell Research Centre, Vancouver, Canada).

Transfection of BOSC23 Cells and Generation of A20-IIA1.6 Cells Expressing Chimeric Receptors. Transfection of BOSC23 cells was performed as described (19). The virus containing supernatant was passed through a 0.2-μm filter, mixed with polybrene to a final concentration of 5 μg/ml and added to 2×10^6 A20-IIA1.6 cells. The retrovirally transduced cells were incubated for 4 days in medium containing puromycin (1.5 μg/ml) followed by fluorescence-activated cell sorter (FACS; Becton Dickinson) sorting to eliminate cells that expressed only GFP or the chimeric receptor.

Cellular Activation, Western Blotting, Affinity Precipitation, and Immunoprecipitation. To monitor the effects of the transduced receptors on BCR-induced signaling, 5×10^6 cell aliquots were washed twice and incubated for 2 h in medium lacking FCS and supplemented with 20 mM Hepes (pH 7.2), before stimulation with intact anti-IgG immunoglobulins (25 μg/ml) or anti-IgG F(ab′)$_2$ fragments (16.6 μg/ml). Western blotting and immunoprecipitations were performed as described (19). Briefly, cells were spun down before incubation in lysis buffer containing 1% Nonidet P-40, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, and the addition of protease inhibitors leupeptin (5 μg/ml), pepstatin (1 μg/ml), aprotinin (5 μg/ml), PMSF (40 μg/ml), and phosphatase inhibitors Na_3VO_4 (0.2 mM), Na_2MoO_4 (1 mM), and β-glycero-phosphate (5 mM). The whole cell lysate was treated with 4 μg of anti-HA antibody 12CA5, followed by addition of 30 μl of a 50% slurry of protein G beads (Amersham Pharmacia Biosciences) and incubation at 4°C for 30 min. The beads were washed three times with 1 ml of lysis buffer and boiled, and the immunoadsorbed proteins were resolved by SDS/PAGE before transfer to nitrocellulose membranes (Schleicher and Schüll, Kassel, Germany), which were probed with the indicated antibodies and visualized by using the ECL reagent (Amersham Pharmacia Biosciences). Whole cell lysate proteins were quantitated by using the BCA reagent (Pierce).

Calcium-Flux Assays. Cells (5×10^6) were washed twice in Hanks' balanced salt solution (HBSS) (with Ca^{2+} and Mg^{2+}), then resuspended in 1 ml of HBSS followed by addition of Fluo-4 AM and SNARF-1 (Molecular Probes) to concentrations of 2 μM and 4 μM, respectively. After incubation for 30 min at 37°C, the cells were washed twice in HBSS and resuspended in 2.5 ml HBSS. Five hundred-microliter aliquots of the cells were used for fluorescence-activated cell sorter analysis after addition of 25 μg/ml intact anti-IgG or 16.6 μg/ml F(ab′)$_2$ fragments of anti-IgG.

Quantitative Analysis of FcRH4 mRNA Expression Levels. Tonsillar mononuclear cells were isolated by a Ficoll-Hypaque gradient centrifugation step, stained for cell surface antigens with combinations of monoclonal antibodies, and sorted into subpopulations with a MoFlow instrument (Cytomation, Fort Collins, CO). Markers used to identify discrete subpopulations were: $CD27^-$/$CD38^-$/IgD^+/$CD19^+$ (naive B cells), $CD38^+$/IgD^+CD19^+ (preGC cells), $CD77^+$/$CD38^+$/$CD19^+$ (centroblasts), $CD77^-$/$CD38^+$/$CD19^+$ (centrocytes), $CD27^+$/$CD38^-$/$CD19^+$ (memory B cells), and $CD38^{2+}$/IgD^-/$CD19^+$ (plasma cells). Sorted cells were lysed in TRIzol reagent (GIBCO), and first-stand cDNA synthesis was performed by using random hexamers and the Superscript II system (Invitrogen). Quantitative analysis of FcRH4 mRNA was performed by real-time PCR by using an ABI Prism 7900 HT sequence detection system. Primers were designed to overlap an exon-intron border to avoid amplification of potential genomic DNA contamination, and PCR reactions were performed by using SYBR Green PCR Master Mix (Applied Biosystems). The values obtained for FcRH4 were normalized to GAPDH expression.

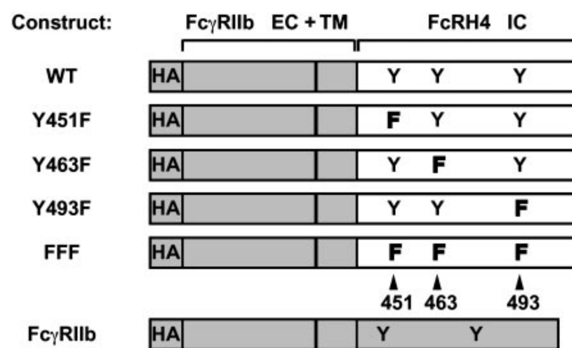

Fig. 1. Schematic illustration of the FcγRIIb and FcγRIIb/FcRH4 chimeric constructs. Portions corresponding to FcγRIIb are indicated by gray shading. Tyrosines of the potential FcRH4 ITIMs and their positions corresponding to full-length FcRH4 are indicated. Tyrosine to phenylalanine mutations are indicated by the letter F (in bold).

Results

Rapid Tyrosine Phosphorylation of FcRH4 After Coligation with the BCR. To investigate the signaling potential of FcRH4, we generated chimeric proteins consisting of the extracellular and transmembrane domains of FcγRIIb fused to the intracellular domain of FcRH4. Expression of these chimeric proteins by cells of the IgG-expressing A20-IIA1.6 B cell line, which lack endogenous FcγRIIb, allowed us to monitor the effects of the intracellular domain of FcRH4 on coligation of the chimeric receptor with the BCR. The bridging of the BCR and the FcγRIIb/FcRH4 chimera was accomplished by the binding of intact anti-Ig antibody to the FcγRIIb portion of the chimeric molecule while unembellished BCR signaling was monitored by treatment of the cells with F(ab′)$_2$ fragments of the anti-IgG antibodies. For comparison with the known inhibitory effects of FcγRIIb, the same experiments were performed with A20-IIA1.6 cells expressing the full-length FcγRIIb. In addition, tyrosine to phenylalanine mutations of potential ITIMs in the cytoplasmic domain of FcRH4 were introduced by site-directed mutagenesis (Fig. 1), and polyclonal populations of cells expressing comparable levels of the chimeric receptors were generated (see Fig. 7, which is published as supporting information on the PNAS web site).

Crosslinking of the WT unmutated FcγRIIb/FcRH4 chimeric protein alone did not result in detectable tyrosine phosphorylation (data not shown). However, coligation of the WT FcγRIIb/FcRH4 receptor with the BCR resulted in tyrosine phosphorylation of the chimeric protein; the tyrosine phosphor-

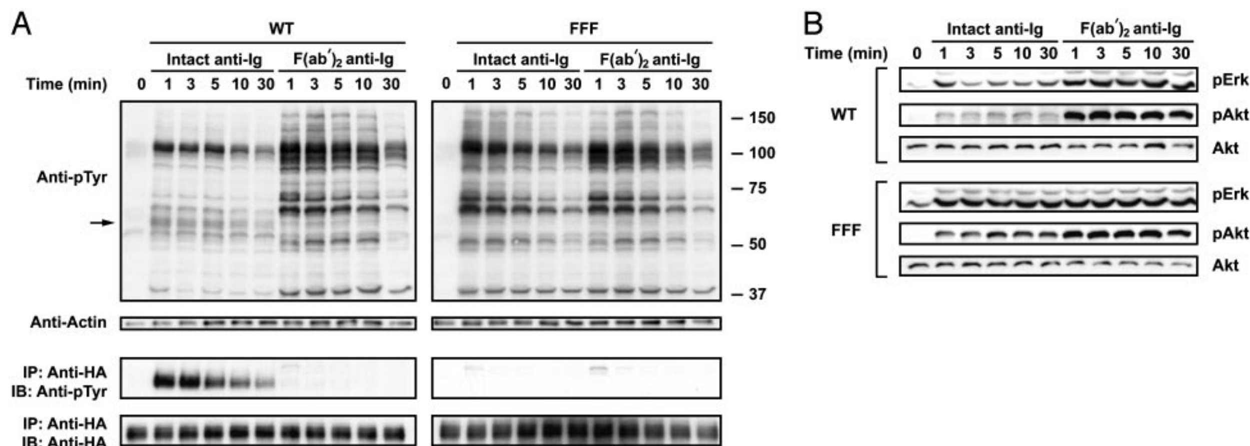

Fig. 2. Coligation of the WT FcγRIIb/FcRH4 chimeric receptor inhibits BCR-induced tyrosine phosphorylation of multiple intracellular proteins, including Erk and Akt. (*A*) WT or FFF cells (5×10^6) were stimulated with intact anti-IgG (25 μg/ml) or $F(ab)_2$ fragments of anti-Ig antibodies (16.6 μg/ml) for the indicated time intervals before analysis of whole cell tyrosine phosphorylation or tyrosine phosphorylation of the immunoprecipitated chimeric receptors. Whole cell lysates (*Upper*) and immunoprecipitates (*Lower*) were analyzed by using anti-phospho tyrosine antibody 4G10. The blots were stripped and reprobed with anti-actin antibody (whole cell lysates) and anti HA-tag antibody (immunoprecipitates), respectively, to assure equal loading of the lanes. Arrow indicates the position of the chimeric receptor. (*B*) Whole cell lysates from *a* were separated by SDS/PAGE, and the blot was cut in half at the 50-kDa marker. *Upper* was probed with anti-phospho Akt and *Lower* with anti-phospho Erk antibodies. Equal loading was verified by reprobing with anti-Akt antibodies.

ylation reached maximal levels 1–3 min after addition of the intact anti-BCR antibodies (Fig. 2*A*). This event was accompanied by a striking reduction of whole cell tyrosine phosphorylation, and specifically of Erk and Akt phosphorylation (Fig. 2*B*). In keeping with earlier studies (20), coligation of FcγRIIb with the BCR also reduced Erk and Akt activation, while having no demonstrable effect on whole cell tyrosine phosphorylation (data not shown). In contrast, the mutant FFF chimera, in which all three tyrosines of the FcRH4 cytoplasmic region were mutated to phenylalanines, had no effect on BCR-induced whole cell tyrosine phosphorylation (Fig. 2*A*) or Erk activation although a slight reduction of Akt activation was observed (Fig. 2*B*).

FcRH4 Completely Inhibits BCR-Induced Calcium Flux. Having determined a potent effect of the FcγRIIb/FcRH4 chimeric protein on BCR-induced tyrosine phosphorylation, as well as on Erk and Akt activation, we compared the effects of full-length FcγRIIb and the FcγRIIb/FcRH4 chimeric receptors on BCR-induced calcium flux. Whereas BCR ligation with $F(ab')_2$ anti-Ig resulted in a characteristic calcium flux, this response was completely abrogated when the cells were stimulated with intact anti-Ig antibodies to coligate the BCR and the WT FcγRIIb/FcRH4 receptor (Fig. 3). This potent inhibitory effect was in striking contrast to the incomplete inhibition mediated by FcγRIIb coligation with the BCR. In control experiments, BCR stimulation with both the $F(ab')_2$ anti-Ig and the intact anti-Ig antibodies elicited a normal calcium mobilization in control cells transduced with "empty vector."

The above experiments were performed in parallel with other experiments designed to identify the tyrosine residues needed for the inhibitory activity of the FcRH4 cytoplasmic region. Stimulation of cells expressing the Y451F mutant with intact anti-Ig antibodies completely blocked the BCR-induced calcium mobilization, indicating that this tyrosine residue is not essential for the inhibitory activity of FcRH4. In contrast, cells expressing the Y463F or Y493F mutants displayed partial inhibition of the calcium flux assay, indicating the importance of these residues

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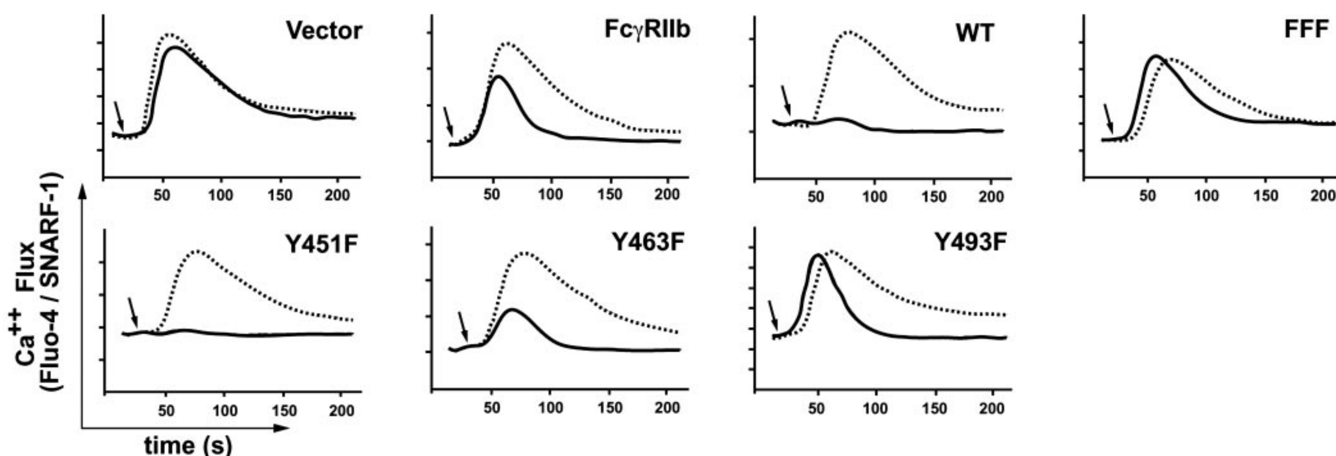

Fig. 3. FcγRIIb/FcRH4 WT coligation inhibits the BCR-induced calcium flux. The indicated cell populations were loaded with the dyes Fluo-4 and SNARF-1 to evaluate calcium levels and stimulated with 25 μg/ml intact anti-IgG antibodies (solid line) or 16.6 μg $F(ab)_2$ fragments of anti-IgG antibodies (dotted line). The arrow indicates the point of addition of the stimulating antibodies. Shown is one of four independent experiments giving the same result.

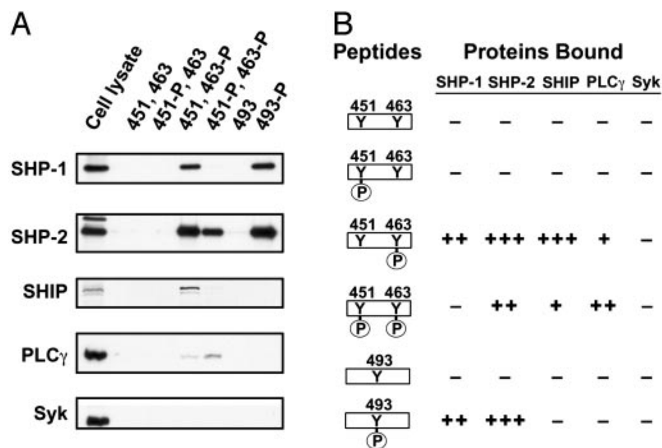

Fig. 4. Phosphorylated peptides corresponding to FcRH4 ITIMs associate with SH2 domain-containing proteins. (*A*) Cell lysates from unstimulated A20IIA1.6 cells were incubated with the indicated peptides, and peptide precipitates were blotted with the indicated antibodies. One of four experiments with the same outcome is shown. (*B*) Graphic illustration of the experimental results. −, No detectable binding; +, weak binding; ++, intermediate binding; +++, strong binding.

for complete FcRH4-mediated inhibition of BCR-signaling (Fig. 3). The nature of the inhibition was different for the Y463F and Y493F mutants, however. Whereas the Y463F mutant inhibited both maximal intensity and duration of the BCR triggered calcium flux, the Y493F mutant abbreviated the duration of the calcium signal while having less effect on its peak intensity (Fig. 3). Collectively, these results indicate that the two membrane-distal ITIMs are pivotal for the potent inhibitory capability of the FcRH4 cytoplasmic domain.

Phosphopeptides Corresponding to the Consensus FcRH4 ITIMs Bind Signaling Proteins with SH2 Domains. Given that the intracellular domain of FcRH4 can inhibit protein tyrosine phosphorylation, Erk and Akt activation, and calcium mobilization, we sought to identify the proteins that mediate this inhibitory effect. In this analysis, phosphopeptides corresponding to the consensus ITIMs of FcRH4 were used as affinity reagents to search for proteins in cell lysates of A20-IIA1.6 cells that could associate with the synthetic ligands. Because the tyrosines 451 and 463 potentially could be components of two separate ITIMs or of a single noncanonical immunoreceptor tyrosine-based activation motif (ITAM), a peptide extending from FcRH4 residues 438 to 472 was synthesized to encompass this entire region. Different versions of this peptide were phosphorylated on tyrosine residue 451, tyrosine residue 463, or both of these tyrosine residues. No proteins were found to bind to this peptide in its unphosphorylated form or when singly phosphorylated at tyrosine 451 (Fig. 4*A*). However, the two tyrosine phosphatases SHP-1 and SHP-2 bound equally well to the peptide that was singly phosphorylated at tyrosine residue 463. The peptide phosphorylated on tyrosine residue 463 was also capable of associating with the inositol phosphatase SHIP. Interestingly, when this peptide was doubly phosphorylated at tyrosine residues 451 and 463, it retained the capacity for SHP-2 binding, lost affinity for the SHP-1 and SHIP phosphatases, and gained the ability to bind PLCγ2 (Fig. 4*A*). SHP-1 and SHP-2 were also found to associate with the peptide phosphorylated at tyrosine 493. The SH2-domain containing Syk tyrosine kinase failed to bind to any of the phosphorylated or nonphoshorylated peptides. Proteins capable of associating with the phosphorylated ITIM candidates in the intracellular domain of FcRH4 are summarized in Fig. 4*B*, the most dramatic association being SHP-1 and SHP-2 with the membrane-distal ITIMs.

SHP-1 and SHP-2 Bind to the FcγRIIb/FcRH4 Chimeric Receptors When Coligated with BCR. To determine which of the phosphopeptide-associated proteins interact with the tyrosine-phosphorylated intracellular domain of the FcγRIIb/FcRH4 chimeric receptors after BCR coligation, cells expressing the different forms of FcRH4 or FcγRIIb were stimulated with intact anti-Ig antibodies. After BCR coligation, immunoprecipitates of the FcRH4 chimeric proteins were probed with either anti-phosphotyrosine antibodies or antibodies specific for SHP-1, SHP-2, or SHIP. The WT FcγRIIb/FcRH4 and Y451F mutant were tyrosine phosphorylated to a similar extent after coligation with the BCR (Fig. 5*A*), as would be expected from the similar effects of this mutant and the WT chimeric receptor on BCR-induced calcium flux. Compared with the WT chimera, the phosphotyrosine signals of the Y463F and Y493F mutants were significantly reduced after coligation with the BCR, and the FFF mutant yielded no detectable phosphotyrosine signal. When the immunoprecipitates were probed for associating proteins, SHP-1 and SHP-2 were coprecipitated with WT FcγRIIb/FcRH4 and the Y451F mutant, but not with the other mutants. In contrast, FcγRIIb immunoprecipitates contained SHIP (Fig. 5*A*) as was anticipated from earlier studies (21). PLCγ could not be detected in any of the immunoprecipitates (data not shown). The similarity of the phosphotyrosine signals observed for the WT FcγRIIb/FcRH4 and the Y451F mutant plus the ability of both proteins to bind SHP-1 and SHP-2 suggest that the membrane-distal ITIMs are sufficient for the inhibitory effect of FcRH4 on BCR-induced signaling events. In contrast, tyrosine 451 in the membrane-proximal ITIM candidate is dispensable.

FcγRIIb/FcRH4-Mediated Inhibition of Erk Activation Correlates with the SHP-1 and SHP-2 Association and Inhibition of BCR-Induced Calcium Flux. We next investigated the effect of the WT and mutant FcγRIIb/FcRH4 chimeric proteins for their ability to inhibit Erk activation. The WT FcγRIIb/FcRH4 and mutant Y451F chimeras, both of which associate with SHP-1 and SHP-2, inhibited Erk phosphorylation after BCR coligation. In comparison with the WT or mutant Y451F chimeras, coligation of the Y463F mutant only moderately attenuated Erk activation (Fig. 5*B*). No effect was observed on Erk activation when the FFF mutant was coligated with the BCR, and a minimal effect was observed for the Y493F mutant. The capacity of the different FcγRIIb/FcRH4 chimeras to modify BCR-induced Erk activation thus faithfully reflects their effects on BCR-mediated calcium flux.

FcRH4 Is Expressed Predominantly by Memory B Cells. A B cell-specific pattern of expression for *FcRH4* mRNA has been indicated in previous studies (14, 15). To refine the definition of the *FcRH4* mRNA expression pattern during B cell differentiation, we examined the *FcRH4* transcript levels as a function of B cell differentiation. Tonsillar B cells were sorted into subpopulations representative of different stages of differentiation based on their expression levels of CD38 and IgD (22, 23). Determination of *FcRH4* transcript levels by real-time PCR indicated that *FcRH4* mRNA is preferentially expressed in the $IgD^-/CD38^-$ subpopulation of memory B cells (Fig. 6). Much lower levels of *FcRH4* mRNA were detected in the naive B cell, pregerminal center, and centroblast subpopulations whereas *FcRH4* expression could not be detected in centrocytes and plasma cells.

Discussion

Remarkably different signaling capabilities of two inhibitory receptors expressed by B cells, FcγRIIb and FcRH4, were demonstrated in these studies. For a direct comparison of the

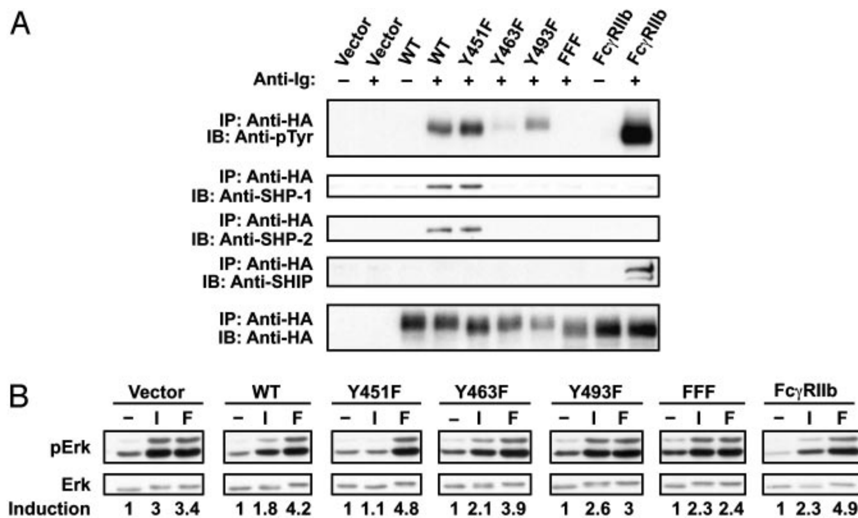

Fig. 5. Correlation of the intracellular association of phosphorylated FcγRIIb/FcRH4 chimeric protein with SHP-1, SHP-2, and Erk activation. (*A*) Evaluation of WT and mutant FcγRIIb/FcRH4 association with SHP-1 and SHP-2. A20IIA1.6 cells transduced with the indicated constructs were stimulated (+) with intact anti-IgG antibodies for 3 min or left untreated (−) before cell lysis and immunoprecipitation with the anti-HA tag antibody 12CA5. The immunoprecipitates were separated by SDS/PAGE and blotted with the indicated antibodies (top four blots). The blot was then stripped and reprobed with the anti-HA tag antibody 12CA5 to assure equal loading (bottom blot). (*B*) Evaluation of Erk phosphorylation status. Cell lysates of the various mutants were left untreated (−) or stimulated with intact anti-IgG antibodies (I) or $F(ab)_2$ fragments of anti-Ig antibodies (F) for 15 min. The lysates were probed with anti-phospho Erk antibodies (*Upper*) then stripped and reprobed with non-phospho-specific Erk antibodies (*Lower*) to verify equivalent protein loading. Erk induction was measured by densitometry and indicated as fold induction over the corresponding untreated control.

signaling potential of the FcRH4 and FcγRIIb cytoplasmic regions, B cells lacking these receptors were transduced with constructs for FcγRIIb and for chimeric proteins consisting of the extracellular and transmembrane domains of FcγRIIb fused to the FcRH4 cytoplasmic domain. Both of these receptors, which contain consensus ITIMs, could then be coligated with the BCR by treating the cells with intact anti-Ig antibodies, whereas $F(ab')_2$ fragments of the anti-Ig antibodies triggered unadulterated B cell responses. In this "tail to tail" comparison, FcRH4 exhibited much greater inhibitory capacity than FcγRIIb, the classical inhibitory receptor for B cells.

When coligated with BCR, the WT FcγRIIb/FcRH4 chimera exerted a strong inhibitory effect on BCR signaling that was characterized by greatly diminished (*i*) whole cell tyrosine phosphorylation, (*ii*) Erk and Akt activation, and (*iii*) calcium mobilization. Although FcγRIIb is well known to inhibit BCR signaling, the mechanism for the two inhibitory activities seems to be quite different. FcγRIIb down-regulates, but does not abolish, BCR signaling through recruitment of the inositol phosphatase SHIP whereas the FcRH4 intracellular domain was found to associate with the SHP-1 and SHP-2 tyrosine phosphatases and to virtually extinguish BCR-mediated signaling. Because the Igα and Igβ components of the BCR are among the substrates of SHP-1 (24), the FcRH4-mediated inhibition of BCR signaling via the SHP-1 and SHP-2 phosphatases may occur through dephosphorylating these BCR-associated signaling chains. In contrast, FcγRIIb recruits the SHIP inositol phosphatase, which regulates BCR signaling further downstream, thus having a weaker effect on BCR signaling. The differences between the FcRH4 and FcγRIIb signaling modes also suggest that these receptors could perform different functions when coexpressed on the same cell.

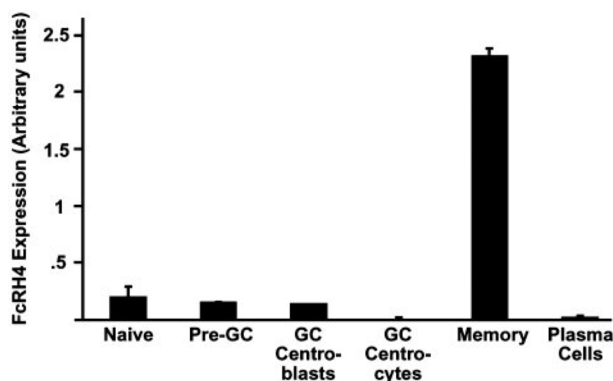

Fig. 6. Real-time PCR analysis of FcRH4 mRNA expression levels as a function of B cell differentiation status. The indicated subpopulations of tonsillar B cells were purified by fluorescence-activated cell sorter. Random, primed cDNA corresponding to 1,000 cells was used for each PCR reaction, and the FcRH4 values obtained were normalized to GAPDH expression. Mean values ± SD ($n = 6$) are shown.

Mutational analysis of the three ITIM candidates in the FcRH4 intracellular domain indicates the functional importance of the two membrane-distal ITIMs, positioned at tyrosine residues 463 and 493, in regulating BCR signaling. Although phosphopeptides corresponding to the two membrane-distal ITIMs could individually precipitate SHP-1 and SHP-2, functional studies, including analysis of calcium mobilization and Erk activation, and coimmunoprecipitation experiments suggest that both ITIMs are simultaneously involved in negatively regulating BCR signaling. This finding implies involvement of both of the two SH2-domains of SHP-1 and/or SHP-2 in binding to the two membrane-distal ITIMs. Mutation of either one of these ITIMs might thus result in lower affinity binding of the phosphatase and consequently lead to the observed impaired function of the cytoplasmic region of FcRH4. It has been demonstrated that the N-terminal SH2-domain of SHP-1 requires a leucine residue in the −2 position relative to the phosphorylated tyrosine for high affinity binding (25, 26), and the second ITIM of FcRH4 matches this sequence requirement. Currently ongoing experiments in DT40 cells with targeted disruption of the SHP-1 and SHP-2 genes will clarify the relative importance of these tyrosine phosphatases.

The two membrane-proximal ITIMs of the FcRH4 intracellular domain could jointly form a noncanonical immunoreceptor tyrosine-based activation motif. Analysis of the degree of tyrosine phosphorylation of the FcRH4 intracellular domain after BCR coligation revealed no differences between the WT FcRH4 form and the version in which the tyrosine of the membrane-proximal ITIM candidate (Y451F) was mutated. This result suggests that tyrosine 451 is not phosphorylated in response to coligation with the BCR. However, a synthetic peptide with phosphorylated tyrosine residues 451 and 463 was capable of associating with PLCγ, a signaling molecule frequently associated with activating rather than inhibitory function. This finding raises the interesting possibility that, in a different signaling context, FcRH4 could become phosphorylated on tyrosines 451 and 463 to engage a different set of effector proteins and assume an activating function.

FcRH4 has previously been shown to be preferentially expressed by B lineage cells (14, 15), and the present analysis indicates that *FcRH4* is expressed primarily by memory B cells. This remarkably discrete pattern of *FcRH4* expression accords with the recent report (27) that the IRTA1/FcRH4^{+} cells are located principally in the perifollicular and subepithelial regions of human tonsils, sites rich in memory B cells (28), and may express the CD27 memory B cell marker. In addition to the high levels of *FcRH4* mRNA in memory cells, we also detected *FcRH4* expression in the IgD^{+}/CD38^{-} "naïve" B cell subpopulations, although at relatively low levels. This finding could reflect memory B cell contamination because up to 37% of the IgD^{+}/CD38^{-} subpopulation may be IgD-bearing memory B cells (23).

Sequence homology with the Fc Ig-binding regions of the classical Fc receptors FcγRI, FcγRIIb, and FcεR provided the basis for identification of FcRH4 in our laboratory (14). Moreover, comparison with proteins of known structure by using the three-dimensional position-specific scoring matrix database (29) indicates that the two membrane-distal Ig domains of FcRH4 are most homologous to the Ig domains of the low-affinity IgG receptor FcγRIIb. However, experimental verification of Ig-binding capability for FcRH4 will be required to assess its physiological potential for modulating antibody responses. As a molecule capable of transmitting a potent inhibitory signal, FcRH4 may also provide an attractive target for the treatment of B cell lymphomas of memory cell phenotype.

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