Src Homology 2 Domain-containing Inositol 5-Phosphatase 1 Mediates Cell Cycle Arrest by FcγRIIB*

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We previously found that low affinity receptors for the Fc portion of IgG, FcγRIIB, which are widely expressed by hematopoietic cells, can negatively regulate receptor tyrosine kinase-dependent cell proliferation. We investigated here the mechanisms of this inhibition. We used as experimental models wild-type mast cells, which constitutively express the stem cell factor receptor Kit and FcγRIIB, FcγRIIB-deficient mast cells reconstituted with wild-type or mutated FcγRIIB, and Src homology 2 domain-containing inositol polyphosphate 5-phosphatase 1 (SHIP1)-deficient mast cells. We found that, upon coaggregation with Kit, FcγRIIB are tyrosyl-phosphorylated, recruit SHIP1, but not SHIP2, SH2 domain-containing protein tyrosine phosphatase-1 or -2, abrogate Akt phosphorylation, shorten the duration of the activation of mitogen-activated protein kinases of the Ras and Rac pathways, abrogate cyclin induction, prevent cells from entering the cell cycle, and block tyrosine incorporation. FcγRIIB-mediated inhibition of Kit-dependent cell proliferation was reduced in SHIP1-deficient mast cells, whereas inhibition of IgE-induced responses was abrogated. Cell proliferation was, however, inhibited by coaggregating Kit with FcγRIIB whose intracytoplasmic domain was replaced with the catalytic domain of SHIP1. These results demonstrate that FcγRIIB use SHIP1 to inhibit pathways shared by receptor tyrosine kinases and immunoreceptors to trigger cell proliferation and cell activation, respectively, but that, in the absence of SHIP1, FcγRIIB can use other effectors that specifically inhibit cell proliferation.

FcγRIIB are widely expressed single-chain low affinity receptors for the Fc portion of IgG antibodies that bind multivalent immune complexes with high avidity. They exist as two mental models wild-type mast cells, which constitutively based activation motifs (ITAMs), murine and human FcγRIIB were shown to negatively regulate cell activation. Thus, FcγRIIB inhibit BCR-mediated B cell activation (5–7), T cell receptor-mediated T cell activation (3) and high affinity IgE receptor (FcεRI)-mediated mast cell activation (3, 8). Long ago, B cell FcεR, which were later identified as FcγRIIB (9), were also shown to inhibit BCR-mediated B cell proliferation (6), and we found recently that murine FcγRIIB can negatively regulate the proliferation of mast cells induced by Kit (10). Kit is a typical receptor tyrosine kinase for stem cell factor (SCF) that belongs to the colony-stimulating factor-1/platelet-derived growth factor receptor subfamily (11) and controls cell proliferation during gametogenesis, melanogenesis, and hematopoiesis (12). The present work aimed at elucidating the mechanism(s) by which FcγRIIB could inhibit cell proliferation.

Works published during the last 5 years documented the molecular mechanisms used by murine FcγRIIB to negatively regulate cell activation triggered by ITAM-bearing receptors. The inhibitory properties of FcγRIIB were found to depend on an immunoreceptor tyrosine-based inhibition motif (ITIM) present in the intracytoplasmic (IC) domain of all murine and human isoforms of FcγRIIB (3, 13). The coaggregation of FcγRIIB with activating receptors enables the Src family protein tyrosine kinase Lyn to phosphorylate not only ITAMs but also the FcγRIIB ITIM (14). The tyrosyl-phosphorylated ITIM recruits the SH2 domain-containing inositol phosphate 5-phosphatase 1 (SHIP1) (15, 16), which inhibits two major signaling pathways triggered by ITAM-bearing receptors, the Ca2+ response and the Ras pathway. The preferred substrate of SHIP1 is indeed phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3), generated by phosphatidylinositol 3-kinase (PI3K) (17). PI(3,4,5)P3 mediates the membrane translocation of a subset of molecules containing a pleckstrin homology (PH) domain. Among these molecules is the Bruton’s tyrosine kinase (18), which is mandatory for phospho-

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††The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; BCR, B cell receptor for antigen; BMMC, bone marrow-derived mast cells; GAM, goat anti-mouse Ig; HRP, horseradish peroxidase; IC, intracytoplasmic; ITIM, immunoreceptor tyrosine-based inhibition motif; MAP, mitogen-activated proteins; FE, phycocyanin; PH, pleckstrin homology; PI(3,4,5)P3, phosphatidylinositol (3,4,5)-trisphosphate; PI3K, phosphatidylinositol-3-kinase; PY, phosphotyrosine; RAM, rabbit anti-mouse Ig; SH2, Src homology 2; SCF, stem cell factor; SHIP, SH2 domain-containing inositol polyphosphate 5-phosphatase; SHP, SH2 domain-containing protein tyrosine phosphatase; wt, wild-type; JNK, c-Jun NH2-terminal kinase; mAb, monoclonal antibody; FcγRIIB(1C1), IC domain-deleted FcγRIIB; IRES, internal ribosomal entry sequence; EGFP, enhanced green fluorescence protein; TNF, tumor necrosis factor; biotin-Ack2, biotinylated anti-Ki ACK2.
lipase C-γ (19) to be activated and to generate inositol 1,4,5-trisphosphate, leading to the mobilization of intracellular Ca^{2+}. SHP1 was recently found to inhibit the activation of Erk1/2, the mitogen-activated protein (MAP) kinases of the Ras pathway, independently of its phosphatase activity. When recruited by FcγRIIB, SHP1 is tyrosyl-phosphorylated and serves as an adapter protein. It recruits p62dok, which is in turn tyrosyl-phosphorylated and recruits RasGAP. RasGAP activates the autocatalytic GTPase activity of ras, thereby preventing the activation of the ras pathway. SHP1 therefore appears as the major effector of FcγRIIB-dependent negative regulation of cell activation by acting at different steps of signal transduction via phosphatase activity-dependent and -independent mechanisms. As a consequence, the activation of Ca^{2+}-dependent enzymes that promote the nuclear translocation of the nuclear factor of activation NF-AT is prevented, as well as MAP kinase-dependent downstream events. MAP kinase substrates are transcription factors that cooperate with NF-AT to induce the transcription of cytokine genes (20, 21). By recruiting SHP1, FcγRIIB therefore arrest the intracellular propagation of activation signals triggered by ITAM-bearing receptors and subsequent cellular responses. These include exocytosis, in mast cells (3, 8), and cytokine secretion, in mast cells (8), B cells (7) and T cells (3).

Mechanisms used by FcγRIIB to inhibit cell proliferation are poorly understood. They are difficult to examine in B cells. Indeed, B cell activation and proliferation can both be triggered by the BCR, which is constitutively associated with several coreceptors whose respective roles in B cell activation and proliferation are not well known. Another reason is that most biochemical studies that unraveled the inhibitory mechanisms used by FcγRIIB were conducted in transformed cell lines whose proliferation became independent of the regulatory mechanisms that control the growth of normal cells. By contrast with B cells, the activation and proliferation of mast cells can be triggered independently by FcεRI and by Kit, respectively (22). Evidence that FcγRIIB can negatively regulate the proliferation of Kit-induced mast cell proliferation originated from our observation that anti-Kit antibodies could induce the proliferation of primary mast cells derived in vitro from mouse bone marrow (BMMCs) provided that their Fc portions could not bind to FcγRIIB that are constitutively expressed by these cells. Comparable proliferative responses were induced by F(ab′)_2 fragments of anti-Kit antibodies in wt mast cells, by intact anti-Kit antibodies in FcγRIIB−/− mast cells, or by intact anti-Kit antibodies in wt mast cells whose FcγRIIB were blocked with anti-FcγRIIB antibodies. No proliferation was observed if anti-Kit antibodies were allowed to coaggregate Kit with FcγRIIB on wt mast cells (10). FcγRIIB therefore inhibit mouse mast cell proliferation when coaggregated with Kit, and BMMCs provide an appropriate model to study the effects of FcγRIIB on signal transduction pathways leading to cell proliferation.

When dimerized by SCF, Kit autophosphorylates and recruits several kinases including PI3K. By generating PI[3,4,5]P_3, PI3K enables the membrane translocation of the protein kinase Akt and the exchange factor Vav via their PH domain. These molecules, altogether, prevent cell death and activate the Rac pathway whose terminal effectors are the MAP kinases p38 and JNK (23). Kit also recruits Shc, which, when phosphorylated, recruits the adapter protein Grb2. Grb2 recruits the exchange factor Sos, which activates the Ras pathway, whose terminal effectors are the MAP kinases Erk1/2 (24). Rac and Ras MAP kinases shuttle into the nucleus, and they cooperate to activate transcription factors that control the expression of cyclin genes (25). Cyclins are the positive regulatory subunits of a class of protein kinases collectively called cyclin-dependent kinases. These phosphorylate proteins of the retinoblastoma family, leading to the release of the transcription factors E2F, which control the coordinated expression of proteins required for the stepwise progression through the cell cycle (26). In the present study, we provide evidence that, when coaggregated with Kit, FcγRIIB selectively recruit SHP1, inhibit the Ras and the Rac pathways, and prevent cells from entering into the cell cycle by blocking the transcription of cyclin genes. The inhibitory effects of FcγRIIB were partially suppressed in SHP1−/− mast cells and could be mimicked by FcγRIIB, whose IC domain was replaced with the catalytic domain of SHP1.

**Experimental Procedures**

**Antibodies**—The mAb anti-mouse FcγRIIB R9.361 (27) and the mouse IgE mAb 2692-I (28) were used as culture supernatants. The rat mAbs anti-mouse FcγRIIB 2.4G2 (29) and anti-mouse Kit ACK2 (22) were affinity-purified on protein G-Sepharose (Amersham Pharmacia Biotech). ACK2 antibodies were biotinylated as described previously (30). Phycoerythrin (PE)-labeled F(ab′)_2 fragments of goat anti-mouse Ig (GAM), F(ab′)_2 fragments of anti-biotin mAbs, and F(ab′)_2 fragments and intact IgG of polyclonal rabbit anti-mouse Ig (RAM) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), mouse anti-cyclin D2 and D3 antibodies were from NeoMarkers (Union City, CA), rabbit anti-cyclin A antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit anti-phospho-Erk, Erk, phospho-JNK, JNK, phospho-p38, p38, phospho-Akt, and Akt antibodies were from New England Biolabs (Beverly, MA). Rabbit antibodies anti-FcγRIIB IC domain (31) were a gift from Dr. Catherine Saute’s-Fridman (Institut Curie, Paris, France). Rabbit anti-SHIP2 antibodies (32) were a gift from Dr. David Wisniewski (Memorial Sloan-Kettering Cancer Center, New York, NY). Horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine (PY) mAb PY-20 was purchased from Chemicon (Temecula, CA), rabbit anti-SHIP1 antibodies were from Upstate Biotechnology (Lake Placid, NY), mouse monoclonal anti-SHP-1 and anti-SHP-2 were from Transduction Laboratories (Lexington, KY), and HRP-conjugated GAM and goat anti-rabbit Ig antibodies were from Santa Cruz Biotechnology.

**Cells**—BMMCs were obtained by culturing mouse bone marrow cells in RPMI medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (complete medium), and 2% X63-IL3-conditioned medium. After 4 weeks, cultures contained more than 90% mast cells. Culture supernatants were from Life Technologies. cDNA Constructs—cDNA encoding wt FcγRIIB1 and IC domain-deleted FcγRIIB (FcγRIIB1IC1) were described previously (14). cDNA encoding a chimeric molecule made of the extracellular and transmembrane domains of FcγRIIB and, as an IC domain, residues 387–829 of human SHP1, containing the catalytic domain (residues 428–806) was constructed by polymerase chain reaction using the following primers. Sense, 5′-CTG GTA CCG ATG AAG AAC AAG CAC TCA GAG-3′; antisense, 5′-GGG CAG GAG CTC TTC TTA GGC CTC TAA CCG AAG GGC-3′. Kpn1 (GGTACC) and SacI (GAGCTC) sites are underlined. The resulting fragment was cloned at Kpn1 and SacI sites into an NT vector containing sequences encoding the extracellular and transmembrane domains and the six first amino acids of intracytoplasmic domain of FcγRIIB1, under the control of the SV40 promoter and containing a resistance gene to zeocin (NT-zeo) (33). The amplification product was sequenced on the two strains.

**Retroviral-mediated Gene Transfer**—A bicistronic retroviral vector (LZRS-IRES-EGFP) was constructed based on the LZRS-LaZ of Nolan and colleagues (34) in which the LaZ gene was replaced by an IRES (35) fused to the EGFP reporter gene. wt and mutant FcγRIIB were inserted into LZRS-IRES-EGFP at HindIII and EcoRI sites upstream of the IRES sequence. Viral supernatants were produced from transfected Phoenix packaging cells (ATCC, F-14727) after selection for high green fluorescent protein fluorescence. Titers (0.9–2 × 10^5 colony-forming units EGFP/ml) were estimated by infection of 3T3 cells with serial dilutions of virus stocks and measurement of EGFP fluorescence. BMMCs were infected by three rounds of 24-h culture with virus stock. Cells were incubated in the presence of 2% X63-IL3-conditioned medium and 8 μg/ml protamine sulfate on fibronectin-coated plates. 2 days after infection, EGFP+ cells were selected by cell sorting using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA).

**Indirect Immunofluorescence**—Aliquots of 5 × 10^5 BMMCs were incubated for 1 h at 0 °C with K9.361. Cells were washed and stained for 30 min at 0 °C with 50 μg/ml of PE-labeled F(ab′)_2 GAM. Fluorescent
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RESULTS

Anti-Kit Antibodies Can Trigger the Same Signaling Events as SCF—We found previously that anti-Kit antibodies trigger the same proliferative signals as SCF. BMMCs were nonstimulated, stimulated with SCF, or stimulated with anti-Kit immune complexes. In the latter case, cells were preincubated with the rat mAb 2.4G2 that blocks the binding site of FcγRIIB (29), prior to stimulation with preformed immune complexes made of biotinylated anti-Kit ACK2 (biotin-ACK2) and anti-biotin IgG antibodies as described (10). Intraacellular signals and cell responses known to be triggered by SCF were examined when stimulating mast cells under these conditions. Upon dimerization, Kit recruits and activates PI3K (23). Akt activation is a reflection of PI3K-dependent PI(3,4,5)P_3 generation. Kit also recruits adapter proteins that lead to the activation of MAP kinases (24). Akt (Fig. 1A), Erk1/2, JNK, and p38 activation (Fig. 1B) was assessed by examining their phosphorylation by Western blotting 10 min after stimulation. MAP kinases activate transcription factors that promote the expression of cyclin genes (25). The up-regulation of cyclins D2 and D3, and of cyclin A, was assessed by Western blotting 6 and 24 h after stimulation, respectively (Fig. 1C). Cyclins D control the entry of cells into the G1 phase, and cyclin A controls DNA replication and the entry into the G2 phase (26). The percentage of cells in the S + G2-M phases of the cell cycle was assessed by flow cytometry following iodide propidium labeling 24 h after stimulation (Fig. 1D). Thymidine incorporation occurs when cells that have entered the cell cycle reach the S phase. Thymidine incorporation was assessed during a 4-h window, 24 h after stimulation (Fig. 1E). All above responses were triggered by SCF and by biotin-ACK2-anti-biotin complexes. Erk1/2 and p38 activation and cyclin D3 induction were of comparable intensities in response to the two stimuli. Akt phosphorylation and cyclin D2 induction were slightly less intense, and JNK activation and cyclin A induction were clearly less intense when induced by antibodies than when induced by SCF. The proportion of cycling cells was 2-fold lower, and thymidine incorporation was 6-fold lower in response to anti-Kit antibodies than in response to SCF.

Anti-Kit antibodies can therefore trigger the same proliferative signals as SCF, although less efficiently. We next conducted a backward analysis of signaling events that stand upstream of cell division, comparing the effects of aggregating Kit and of coaggregating Kit with FcγRIIB.

When Coaggregated with Kit, FcγRIIB Block the Cell Cycle by Inhibiting Cyclin Expression—We examined the cell cycle and the induction of cyclins, in relation with inhibition of thymidine incorporation, in BMMCs from FcγRIIB<sup>−/−</sup> and FcγRIIB<sup>+/−</sup> mice. FcγRIIB<sup>−/−</sup> BMMCs, but not FcγRIIB<sup>+/−</sup> BMMCs, expressed FcγRIIB as assessed by indirect immunofluorescence with the FcγRIIB-specific mAb K9.361 (Fig. 2A). Kit was aggregated by biotin-Ack2-anti-biotin complexes in FcγRIIB<sup>+/−</sup> cells that were preincubated with 2.4G2 and in FcγRIIB<sup>−/−</sup> cells, whether or not they were preincubated with 2.4G2. Kit was coaggregated with FcγRIIB by the same complexes in FcγRIIB<sup>−/−</sup> cells that were not preincubated with 2.4G2.

Biotin-Ack2-anti-biotin complexes induced thymidine incorporation in FcγRIIB<sup>−/−</sup> BMMCs whether or not they were preincubated with 2.4G2 and in FcγRIIB<sup>−/−</sup> BMMCs that were preincubated with 2.4G2. Biotin-Ack2 complex-induced thymidine incorporation varied similarly in the two cell types with the relative concentrations of biotin-Ack2 and anti-biotin antibodies. Optimal responses were induced by complexes formed at equivalence. No thymidine incorporation was induced in FcγRIIB<sup>−/−</sup> BMMCs that were not preincubated with 2.4G2 (Fig. 2B).

A dose-dependent increase in the percentage of cells in S + G2-M was observed following stimulation of FcγRIIB<sup>−/−</sup>...
BMMCs with biotin-ACK2-anti-biotin complexes, when preincubated with 2.4G2, but not when not preincubated with 2.4G2. A comparable dose-dependent increase in the proportion of Fc/H9253 RIIB/H11002 cells in S/G2M was observed, whether or not cells were preincubated with 2.4G2 (Fig. 2C).

The induction of cyclin D2, cyclin D3, and cyclin A was examined in wt BMMCs following Kit aggregation and following coaggregation of Kit with Fc/H9253 RIIB using the same ligands as above. Kit aggregation increased the intracellular levels of cyclins D2, D3, and A. All three cyclins remained at basal levels following coaggregation of Kit with FcγRIIB (Fig. 2D). FcγRIIB can therefore prevent BMMCs from entering the cell cycle by inhibiting the induction of cyclins.

When Coaggregated with Kit, FcγRIIB Inhibit the Activation of Erk, JNK, p38, and Akt—Kit was aggregated or coaggregated with FcγRIIB for various periods of time in wt BMMCs using the same ligands as in Fig. 2, and the phosphorylation of the MAP kinases Erk1/2, JNKs, and p38 and of the protein kinase Akt were examined. All three MAP kinases were inducibly phosphorylated upon Kit aggregation as early as 3 min after stimulation. Phosphorylation remained at a comparable level at 10 min and decreased at 30 min. The phosphorylation of Erk, JNKs, and p38 occurred normally 3 min after coaggregation of Kit with FcγRIIB but was inhibited at 30 min (Fig. 3). Akt was phosphorylated within 3 min following Kit aggregation, and phosphorylation remained constant until 30 min. Akt phosphorylation was inhibited 3 min after coaggregation of Kit with FcγRIIB and abolished at 30 min (Fig. 3). FcγRIIB therefore shorten the duration of Kit-dependent Erk, JNK, p38, and Akt phosphorylation.

Inhibition of Kit-dependent Cell Proliferation Requires the FcγRIIB Intracytoplasmic Domain—We next analyzed FcγRIIB sequences involved in negative regulation of Kit-dependent proliferation. To this aim, FcγRIIB−/− BMMCs were reconstituted with FcγRIIB1 or with FcγRIIB(IC1) (Fig. 4A). Kit was aggregated or coaggregated with FcγRIIB under the
same conditions as in Fig. 2, and thymidine incorporation was measured. The two types of cells incorporated comparable amounts of thymidine following Kit aggregation. Thymidine incorporation was suppressed when coaggregating Kit with FcγRIIB1 but not with FcγRIIB(IC1) (Fig. 4B). Inhibition of proliferation, which was abolished in FcγRIIB−/− BMMCs, was therefore fully restored following reconstitution with FcγRIIB1 but not with FcγRIIB(IC1).

Biochemical events associated with Kit-dependent proliferation were also examined in the same two BMMCs under the same conditions as in Fig. 3. Kit aggregation induced Erk and Akt phosphorylation at 3 min, and phosphorylation remained unaffected by coaggregating Kit with FcγRIIB(IC1). Akt phosphorylation was dramatically inhibited 3 min and remained abolished 30 min after coaggregation of Kit with FcγRIIB1. It was unaffected by coaggregating Kit with FcγRIIB(IC1) (Fig. 4C). Thus, to inhibit Kit-dependent proliferation, FcγRIIB require the conservation of their IC domain.

Upon Coaggregation with Kit, FcγRIIB Becomes Tyrosyl-phosphorylated and Recruits SHIP1 but Not SHIP2, SHP-1, or SHP-2—Negative regulation of cell activation by FcγRIIB is correlated with the recruitment of SH2 domain-containing phosphatases by tyrosyl-phosphorylated FcγRIIB (15, 16, 38). We investigated which phosphatases were recruited by FcγRIIB upon coaggregation with Kit. FcγRIIB−/− BMMCs reconstituted with FcγRIIB1 were stimulated or not with biotin-ACK2-anti-biotin immune complexes, and FcγRIIB1 were identified by Western blotting with the indicated antibodies. As observed previously,
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SHIP1 Deletion Partially Suppresses FcγRIIB-mediated Inhibition of Kit-dependent Cell Proliferation — We next compared the effects of coaggregating Kit with FcγRIIB on thymidine incorporation in SHIP1−/− and SHIP1+/− BMMCs, using the same conditions as in Fig. 2. Thymidine incorporation induced by Kit aggregation was abolished following coaggregation of Kit with FcγRIIB in SHIP1+/− BMMCs and still inhibited, though only partially, in SHIP1−/− BMMCs (Fig. 7A). This result was unexpected, because SHIP1 deletion was reported to abrogate FcγRIIB-mediated inhibition of BCR-induced cell activation in DT40 cells (40). This led us to investigate the consequences of coaggregating FcγRIIB with FcεRI in SHIP1−/− mast cells. SHIP1−/− and SHIP1+/− BMMCs were sensitized with murine IgE and challenged with F(ab′)2 fragments of RAM antibodies to aggregate FcεRI or with intact RAM IgG antibodies to coaggregate FcεRI with FcγRIIB. Both serotonin release (Fig. 7B) and TNFα secretion (Fig. 7C) induced by FcεRI aggregation were inhibited in SHIP1+/− BMMCs following coaggregation of FcεRI with FcγRIIB. Inhibition of both responses was abolished in SHIP1−/− BMMCs (Fig. 7, B and C).

The residual inhibition of thymidine incorporation in SHIP1−/− BMMCs could be explained if BMMCs underwent apoptosis following coligation of Kit with FcγRIIB. SHIP1−/− was indeed reported to act as an anti-apoptotic molecule in B cells (40, 41). SHIP1−/− and SHIP1+/− BMMCs were therefore cultured for 24 h with medium alone or with biotin-ACK2-anti-biotin complexes after they had been preincubated with or without 2.4G2. Dead cells were visualized with annexin-V and propidium iodide. Comparable proportions of viable cells (80–90%) were observed in the two cell types in all three conditions (Fig. 7D). Apoptosis therefore does not account for residual inhibition of SHIP1−/− BMMC proliferation.

Thus, whereas FcγRIIB-mediated inhibition of FcεRI-dependent cell activation was abolished in SHIP1−/− BMMCs, and although SHIP1 only coprecipitated with FcγRIIB phosphorylated upon coaggregation with Kit in SHIP1+/− BMMCs, FcγRIIB-mediated inhibition of Kit-dependent cell proliferation was only partially abrogated in SHIP1−/− BMMCs. This partial effect correlates with the partial inhibition of cyclin D3 induction observed in SHIP1−/− cells.

The Catalytic Domain of SHIP1 Is Sufficient to Negatively Regulate Kit-dependent Mast Cell Proliferation — The above genetic approach provided negative evidence that SHIP1 is involved in FcγRIIB-mediated negative regulation of Kit-dependent cell proliferation. To obtain direct, positive evidence that SHIP1 can be an effector of this regulation, we constructed a

**Fig. 3. Inhibition of Kit-dependent Erk, JNK, p38, and Akt by FcγRIIB.** BMMCs, preincubated with 2.4G2 (Kit aggregation) or without 2.4G2 (FcγRIIB-Kit coaggregation), were incubated for the indicated periods of time with complexes made of 3 μg/ml biotin-ACK2 (Biot-ACK2) and 3 μg/ml anti-biotin (anti-Biot) antibodies. Cells were lysed, and 5 μg of total proteins for Erk and Akt detection, 25 μg for p38 detection, or 50 μg for JNK detection were electrophoresed and Western blotted with the indicated antibodies.
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Fig. 4. Requirement of FcγRIIB intracytoplasmic sequences for inhibition of Kit-dependent cell proliferation. A, FcγRIIB expression. The binding of the anti-FcγRIIB mAb K9.361 was assessed by indirect immunofluorescence (gray histograms, cells incubated with K9.361 and PE-GAM F(ab′)2; black histograms, cells incubated with K9.361 and PE-GAM F(ab′)2 only). B, thymidine incorporation. FcγRIIB−/− BMMCs reconstituted with FcγRIIB−/− B1, FcγRIIB−/− IC1, or FcγRIIB+IC1 (FcγRIIB−/− IC1), preincubated with 2.4G2 (Kit aggregation; open circles) or without 2.4G2 (FcγRIIB-Kit coaggregation; closed circles) were incubated for 24 h with complexes made of indicated concentrations of biotin-ACK2 and 3 μg/ml anti-biotin antibodies, and thymidine incorporation was measured. The figure represents thymidine incorporation as a function of the concentration of biotin-ACK2. C, Erk and Akt phosphorylation. BMMCs, preincubated with 2.4G2 (Kit aggregation) or without 2.4G2 (FcγRIIB-Kit coaggregation), were incubated for the indicated periods of time with complexes made of 3 μg/ml biotin-ACK2 (Biot-ACK2) and 3 μg/ml anti-biotin (anti-Biot) antibodies. Cells were lysed, and 5 μg of total proteins for Erk detection or 20 μg for Akt detection were electrophoresed and Western blotted with the indicated antibodies.

Fig. 5. Recruitment of SHIP1, but not SHIP2, SHP-1, or SHP-2, by FcγRIIB when tyrosyl-phosphorylated upon coaggregation with Kit. FcγRIIB were immunoprecipitated from BMMCs stimulated for 5 min with medium or with complexes made of 3 μg/ml biotin-ACK2 (Biot-ACK2) and 3 μg/ml anti-biotin (anti-Biot) antibodies. Immunoprecipitates were electrophoresed and sequentially Western blotted with anti-phosphotyrosine antibodies (anti-PY), anti-FcγRIIB antibodies to check that comparable amounts of materials were immunoprecipitated, and anti-SHIP1, anti-SHIP2, anti-SHP-2, and anti-SHP-1 antibodies to identify coprecipitated phosphatases. Whole cell lysates (WCL) were used as positive controls.

cDNA encoding a chimeric molecule made of FcγRIIB whose IC domain was replaced with the catalytic domain of SHIP1. This cDNA was expressed in FcγRIIB−/− BMMCs, and the chimera was compared with FcγRIIB expressed by wt BMMCs for its ability to negatively regulate Kit-dependent proliferation. The expression of the FcγRIIB-SHIP1 chimera was confirmed by indirect immunofluorescence with K9.361 (Fig. 8A). Comparable thymidine incorporation was observed following Kit aggregation in both cells. Thymidine incorporation was similarly inhibited following coaggregation of Kit with wt FcγRIIB or with the FcγRIIB-SHIP1 chimera (Fig. 8B). A SHIP1 chimera could therefore mimic FcγRIIB-mediated negative regulation of Kit-dependent mast cell proliferation.

DISCUSSION

The present work aimed at investigating the mechanism by which FcγRIIB negatively regulate cell proliferation. We used, as an experimental model, Kit-dependent mast cell proliferation that we previously found to be negatively regulated by FcγRIIB (10). We show here that SHIP1 is selectively recruited by tyrosyl-phosphorylated FcγRIIB and plays a critical but not exclusive role in inhibiting transduction pathways that lead to the transcription of cyclin genes and the progression of cells through the cell cycle.

We observed previously a dual effect of IgG anti-Kit antibodies, they can activate cell proliferation by aggregating Kit via their Fab portions, and they can inhibit cell proliferation by coaggregating Kit with FcγRIIB via their Fab and Fe portions (10). We therefore used anti-Kit antibodies to analyze the mechanism of FcγRIIB-dependent inhibition of Kit-induced proliferation. beforehand, we checked that anti-Kit antibodies triggered the same intracellular signals as SCF. When dimerized by SCF, Kit is probably under an optimal spatial configuration on the cell membrane but possibly not when aggregated by anti-Kit antibodies. We found no qualitative difference between signals examined upon stimulation of BMMCs with SCF
and with anti-Kit antibodies, but we did find quantitative differences. Antibodies were indeed less efficient than SCF, and apparently, late signals were more attenuated than early signals following stimulation with antibodies. Whatever the reason of these differences, these preliminary results validated the use of anti-Kit antibodies for studying FcγRIIB-dependent inhibition of signals generated by Kit and leading to mast cell proliferation.

Inhibition of thymidine incorporation correlated with the tyrosyl phosphorylation of FcγRIIB induced upon coaggregation with Kit. Inhibition observed in FcγRIIB−/− BMMCs reconstituted with wt FcγRIIB1 was not seen in FcγRIIB−/− BMMCs reconstituted with an IC domain-deleted FcγRIIB. Inhibition is therefore not a consequence of steric hindrance between extracellular domains and ligands but requires the IC domain of FcγRIIB. Four tyrosines are contained in this domain, one being within the ITIM (3, 13), which when tyrosyl-phosphorylated, has been shown to mediate the recruitment of SHIP1 and the subsequent degradation of PI(3,4,5)P3, enabling the membrane translocation of proteins bearing a PH domain including Akt. Akt phosphorylation, which requires the membrane recruitment of Akt, is a reflection of PI3K activation, and inhibition of Akt phosphorylation is a likely reflection of the catalytic activity of Akt, is a reflection of PI3K activation, and inhibition of Akt phosphorylation is a likely reflection of the catalytic activity of PI3K. Indeed, inhibition of Akt phosphorylation did not occur in SHIP1−/− cells. Interestingly, both SCF-induced and anti-Kit-induced Akt phosphorylation were more intense, particularly at later time points, in SHIP1−/− BMMCs than in SHIP1+/− BMMCs, and FcγRIIB-dependent inhibition of Akt phosphorylation seen in SHIP1+/− cells was more pronounced at the same late time points. When activated, Kit recruits and activates PI3K (23), which generates PI(3,4,5)P3, enabling the membrane translocation of proteins bearing a PH domain including Akt. Akt phosphorylation, which requires the membrane recruitment of Akt, is a reflection of PI3K activation, and inhibition of Akt phosphorylation is a likely reflection of the catalytic activity of PI3K, whose preferred substrate is PI(3,4,5)P3. Similar observations were made in B cells following coaggregation of FcγRIIB with BCR (45, 46). We also found that the phosphorylation of p98 and JNK, the terminal effector MAP kinases of the Rac pathway, was inhibited following coaggregation of Kit with FcγRIIB. This inhibition is also likely due to the recruitment of SHIP1 and the subsequent degradation of PI(3,4,5)P3, which mediates the membrane recruitment of Vav.

Erk1/2 phosphorylation observed following Kit aggregation was of a shorter duration following the coaggregation of Kit with FcγRIIB. This effect was not observed when Kit was coaggregated with an IC domain-deleted FcγRIIB. Inhibition of Erk phosphorylation is also a likely consequence of the recruit-
ment of SHIP1 by tyrosyl-phosphorylated FcγRIIB, because it was prevented in SHIP1\(^{-/-}\) BMMCs. Like Akt phosphorylation, both SCF-induced and anti-Kit-induced Erk phosphorylation lasted longer in SHIP1\(^{-/-}\) BMMCs than in SHIP1\(^{+/+}\) BMMCs, and FcγRIIB-dependent inhibition of Erk phosphorylation seen in SHIP1\(^{+/+}\) cells was more pronounced at late time points. Several mechanisms were proposed to explain how SHIP1 could inhibit immunoreceptor-induced Erk activation. One leads to a decreased production of molecules that activate Ras. Thus, by preventing the Bruton’s tyrosine kinase-dependent full activation of phospholipase C-γ, SHIP1 may decrease the conversion of phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol. Diacylglycerol activates protein kinase C, which activates Ras. Another mechanism consists in the sequestration of molecules that are necessary for the activation of Ras. SHIP1 was indeed reported to compete with Gbr2 for binding Shc, thereby preventing the constitution of the complex of adapters that connect immunoreceptors to the Ras pathway (47). Finally, SHIP1 has recently been shown to inhibit Erk activation by functioning as an adapter molecule. When recruited by FcγRIIB in B cells, SHIP1 is indeed a substrate of Lyn, and when tyrosyl phosphorylated, it recruits Dok via its phosphotyrosine-binding domain. Dok is itself phosphorylated by Lyn and recruits RasGAP via its SH2 domain. RasGAP antagonizes with Sos by accelerating the hydrolysis of GTP into GDP on Ras (48). Kit also recruits adapter proteins that, via the exchange factor Sos, activate the Ras pathway, whose ultimate effectors are the MAP kinases Erk1/2 (24). Whether one or several of these nonexclusive mechanisms account(s) for FcγRIIB-mediated inhibition of Kit-dependent activation of Erk1/2 remains to be investigated.

The induction of cyclins D2, D3 (we found no cyclin D1 in BMMCs), and A observed following Kit aggregation was inhibited when Kit was coaggregated with FcγRIIB. This effect was not observed in FcγRIIB\(^{-/-}\) BMMCs. This inhibition likely results from the inhibition of MAP kinase activation. MAP kinases were indeed shown to control the transcription of cyclin genes (25). Inhibition of cyclin D3 induction was, however, reduced but not suppressed in SHIP1\(^{-/-}\) BMMCs, although inhibition of MAP kinase activation was abolished. This result suggests that, among the mechanisms that control cyclin D3 expression and that are inhibited by FcγRIIB, one can distinguish mechanisms whose inhibition by FcγRIIB is SHIP1-dependent and mechanisms whose inhibition by FcγRIIB is SHIP1-independent.

As a consequence of the inhibition of cyclin induction, the increased proportion of cells entering the S phase observed following Kit aggregation was not seen following the coaggregation of Kit with FcγRIIB. This correlates with inhibition of thymidine incorporation. Expectedly, FcγRIIB-induced inhibition of thymidine incorporation was reduced in SHIP1\(^{-/-}\) BMMCs, but unexpectedly, it was not abolished. This partial...
control of cell proliferation. We found that this regulation depends primarily on the recruitment of SHIP1 by tyrosyl-phosphorylated FcγRIIB when these receptors are coaggregated with Kit by anti-Kit antibodies. Under these conditions, SHIP1 was found to extinguish Kit-induced signals depending on the recruitment of molecules that have a PH domain to the membrane and downstream signals, as well as the activation of the Ras pathway. Notably, signaling pathways triggered by Kit, but not by FcγRI and therefore possibly-specific for cell proliferation, could be inhibited by FcγRIIB in the absence of SHIP1. However, although FcγRIIB could inhibit not only pathways shared by cell activation and cell proliferation, but also pathways specific for cell proliferation, the inhibition of common pathways, by SHIP1, was sufficient to prevent cell proliferation. Our work also provides information on the mechanisms by which SHIP1 negatively regulates signals triggered by Kit. Hematopoietic progenitors from SHIP1+/− mice were indeed reported to be hyper-responsive to several growth factors including SCF (39), and we show here that Akt and Erk phosphorylation were more intense and lasted longer in SHIP1+/− BMMCs than in SHIP1+/+ BMMCs, in response to both anti-Kit antibodies and SCF stimulation. Whether triggered by SCF or by anti-Kit antibodies, Kit-derived signals are under the control of SHIP1 through the inhibition of PI(3,4,5)P3-dependent early events. By recruiting more SHIP1, FcγRIIB may therefore enhance the constitutive negative regulation of Kit signaling by SHIP1.

Based on these results, anti-Kit antibodies may be envisaged as being more than analytical tools. Abnormal cell proliferation may indeed arise from mutations of Kit that render this receptor constitutively activated. A few well characterized oncogenic Kit mutations were found in mastocytosis, mastocytes, mast cell leukemias, and intestinal tumors derived from Cajal interstitial cells, and these mutations are thought to be the etiology of these proliferative diseases. Our results provide the molecular grounds for a potential therapeutic use of anti-Kit antibodies in such malignant diseases.

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Control of Cell Proliferation by FcγRIIB via SHIP1

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