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Running Title: FcγRIIB recruit Grb2/Grap with SHIP1/2

Abstract

FcγRIIB are low-affinity receptors for IgG that contain an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) and that inhibit Immunoreceptor Tyrosine-based Activation Motif (ITAM)-dependent cell activation. When coaggregated with ITAM-bearing receptors, FcγRIIB become tyrosyl-phosphorylated and recruit the SH2 domain-containing phosphatidylinositol 5'-phosphatases SHIP1 and SHIP2, which mediate inhibition. The FcγRIIB ITIM was proposed to be necessary and sufficient for recruiting SHIP1/2. We show here that a second tyrosine-containing motif in the intracytoplasmic domain of FcγRIIB is required for SHIP1/2 to be coprecipitated with the receptor. This motif functions as a docking site for the SH2 domain-containing adapters Grb2 and Grap. These adapters interact *via* their C-terminal SH3 domain with SHIP1/2 to form a stable receptor-phosphatase-adapter trimolecular complex. Both Grb2 and Grap are required for an optimal coprecipitation of SHIP with FcγRIIB, but one adapter is sufficient for the phosphatase to detectably coprecipitate with the receptors. In addition to facilitating the recruitment of SHIPs, the second tyrosine-based motif may confer upon FcγRIIB the properties of scaffold proteins capable of altering the composition and the stability of signaling complexes generated following receptor engagement.

Introduction

FcγRIIB are low-affinity receptors for the Fc portion of IgG antibodies that are widely expressed by cells of hematopoietic origin (1). Their low affinity enables them to remain free in the presence of high concentrations of circulating IgGs and to bind immune complexes with a high avidity. They are unique among Fc Receptors (FcRs) in exhibiting inhibitory properties. Indeed, FcγRIIB were demonstrated to negatively regulate cell activation triggered by other FcRs in mast cells (2), by B Cell Receptors for antigen (BCR) in B cells (3,4) and by T Cell Receptors for antigen (TCR) in T cells (5), *i.e.* by all receptors containing Immunoreceptor Tyrosine-based Activation Motifs (ITAMs). FcγRIIB must be coaggregated with activating receptors *via* IgG immune complexes in order to exert their inhibitory effects (2). The *in vivo* relevance of the regulatory properties of FcγRIIB was ascertained in FcγRIIB-deficient mice. FcγRIIB$^{-/-}$ mice were shown to mount enhanced antibody responses (6), to exhibit enhanced IgG- and IgE-induced anaphylactic reactions (7), to be hypersensitive to collagen-induced arthritis (8,9), and to develop spontaneous systemic *lupus erythematosus* in the C57BL/6 background (10). FcγRIIB are therefore likely to play major roles in the prevention of autoimmune diseases, allergies and other inflammatory diseases.

The regulatory properties of FcγRIIB were shown to depend on the presence of an Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) in their intracytoplasmic domain. This motif was defined as a tyrosine residue, followed, at position Y+3, and preceded, at position Y-2, by hydrophobic aminoacids (11). The ITIM tyrosine becomes phosphorylated by a src family protein tyrosine kinase upon coaggregation of inhibitory receptors with activating receptors (12), providing a docking site for SH2 domain-containing cytosolic phosphatases (13). Due to the presence of a leucine residue at position Y+2 (14), FcγRIIB were shown to recruit selectively the single SH2 domain-containing Inositol 5'-Phosphatases SHIP1 (15,16) and/or SHIP2 (17). These phosphatases dephosphorylate 5'-phosphate groups

in 3'-phosphorylated inositols and phosphatidylinositides (18,19) among which phosphatidylinositol-(3,4,5)-trisphosphate (PI(3,4,5)P3) (20,21), generated by phosphatidylinositol-3 Kinase during cell activation, is a major substrate. PI(3,4,5)P3 enables the membrane translocation of cytosolic molecules possessing Pleckstrin Homology domains, including the Bruton's Tyrosine Kinase, Phospholipase C-γ (PLCγ) and the GTP/GDP exchange factor Vav that are essential for signaling (21). SHIP1 also functions as an adapter to recruit Dok-1 which itself recruits RasGAP, which inhibits Ras activation (22). Taken together, these results suggest that the primary function of FcγRIIB is to recruit SHIP1 (and SHIP2) which dampens positive signaling. Indeed, FcγRIIB-dependent inhibition of cell activation was abrogated in mast cells (23) and markedly inhibited in B cells (24) from SHIP1$^{-/-}$ mice, and inhibition of cell activation could be induced by FcγRIIB whose intracytoplasmic domain was replaced by the catalytic domain of SHIP1 (25,26). Likewise SHIP2, that is inducibly expressed in LPS-activated B cells, may contribute to FcγRIIB-dependent negative regulation of BCR-induced activation of these cells (27).

It is a general consensus that the FcγRIIB ITIM is both necessary and sufficient for inhibition. The conclusion that it is necessary was based on the pioneer work by Amigorena *et al*. who showed that a 13-aminoacid deletion, which was later understood to encompass the ITIM, abrogated inhibition in B cells (4). A point mutation of the ITIM tyrosine also abrogated FcγRIIB-dependent inhibition of mast cell and T cell activation (5), and abolished (28) or reduced (29) the calcium response in B cells. The conclusion that the ITIM is sufficient was based on works by Muta *et al.* who showed that a chimeric molecule whose intracytoplasmic domain contained the murine FcγRIIB ITIM retained inhibitory properties in B cells (28). More recently however, we found that a C-terminal deletion of the intracytoplasmic domain of FcγRIIB, which left the ITIM intact, prevented SHIP1 for being detectably coprecipitated, and reduced the inhibitory effect of FcγRIIB on BCR signaling

(29). We show here that this C-terminal sequence contains a second tyrosine-based motif that mediates the recruitment, *via* their SH2 domain, of the adapter proteins Grb2 and Grap which interact, *via* their C-terminal SH3 domain, with SHIP1 and SHIP2, thus stabilizing the binding of these phosphatases to the FcγRIIB ITIM. Supporting a critical role of this trimolecular complex *in vivo*, we provide evidence that adapters are necessary for FcγRIIB to recruit phosphatases.

Experimental procedures

Cells. IIA1.6 cells were cultured in RPMI supplemented with 10% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 0.5 μM 2-mercaptoethanol and 2 mM sodium pyruvate. DT40 cells (30), purchased from Riken Cell Bank (Tsukuba Science City, Japan), were cultured in RPMI supplemented with 10% FCS, 1% chicken serum 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 0.5 μM 2-mercaptoethanol. Culture reagents were from Life Technologies, Inc. (Paisley, Scotland, UK).

Antibodies. The rat anti-mouse FcγRIIB 2.4G2 mAb (31) was purified on protein G-Sepharose. $F(ab')_2$ fragments and IgG of polyclonal Rat anti-Mouse Ig ($R_{at}AM$), $F(ab')_2$ fragments and IgG of polyclonal Rabbit anti-Mouse Ig ($R_{ab}AM$), IgG of polyclonal Rabbit anti-Chicken Ig ($R_{ab}AC$), FITC-labeled Mouse anti-Rat Ig (MAR_{at}) $F(ab')_2$ and FITC-labeled Goat anti-Rabbit Ig (GAR_{ab}) $F(ab')_2$ were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), rabbit anti-phospho-Akt, anti-Akt, anti-phospho-Erk and anti-Erk antibodies from New England Biolabs (Beverly, MA), mouse anti-Grb2 antibodies from Transduction Laboratories (Lexington, KY), mouse anti-phosphotyrosine mAbs (4G10), rabbit anti-SHIP1, anti-Nck and anti-Nckβ antibodies from Upstate Biotechnology (Lake

Placid, NY), rabbit anti-Grb2 and anti-CrkL antibodies, HRP-conjugated Goat anti-Rabbit (HRP-GAR) and Goat anti-Mouse (HRP-GAM) Ig antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against recombinant EC domains of FcγRIIB and mouse anti-GST antibodies were kind gifts from Pr. C. Sautès-Fridman and Dr. J-L. Teillaud (INSERM U255, Paris, France), respectively. Rabbit anti-SHIP2 antibodies were a gift from Dr. D. Wisniewski (Memorial Sloan-Kettering Cancer Center, New York, NY). Rabbit anti-Grap antibodies were a gift from Dr. G-S. Feng (The Burnham Institute, La Jolla, CA). Rabbit anti-SHIP1 antibodies used in DT40 cells were a gift from Dr. J. V. Ravetch (The Rockefeller University, New York, NY).

cDNA constructs. The cDNA of mouse FcγRIIB was modified by a point mutation of the codon coding for V_{212} (GTT->GTA), which induced no amino acid change, but created a KpnI restriction site. This cDNA encoding the entire extracellular and transmembrane domains and the six first intracytoplasmic amino acids of FcγRIIB was inserted into an expression vector under the control of the SRα promoter in pBR322 (32) and in which a neomycin-resistance gene was introduced. The cDNA encoding the mutated intracytoplasmic domain of FcγRIIB1 Y326F was amplified using the following primers: 5'-Aag AAA Aag Cag gTA CCA gCT CTC CCA-3' and 5'-Cg AgC TCA AAT gTg gAA Ctg AAA ATC Atg CTC TgT TTC TTC-3'. This cDNA was then fused to the cDNA encoding the extracellular and transmembrane domains of FcγRIIB.

Transfectants. cDNAs were stably transfected in IIA1.6 and DT40 cells by electroporation. Transfectants were selected and cloned as described (33-35). Expression of receptors on clones remained stable. Several clones of each transfectant were used and gave similar results.

Indirect immunofluorescence. To measure the expression of FcγRIIB, cells were incubated with 10 μg/ml 2.4G2 or without, washed and stained with 50 μg/ml FITC-labeled MAR_{at} $F(ab')_2$. To measure the expression of BCR, cells were incubated with 10 μg/ml $R_{ab}AC$ or without, washed and stained with 50 μg/ml FITC-labeled GAR_{ab} $F(ab')_2$. Fluorescence was analyzed with a FACScalibur (Becton Dickinson, Mountain View, CA).

Flow cytometric analysis of calcium mobilization. Intracellular free calcium concentration was determined by preloading $1x10^6$ IIA1.6 cells with 5 mM Fluo-3 AM (Molecular Probes, Eugene, OR) in the presence of 0.2 % Pluronic F-127 (Sigma Chemical Co., St. Louis, MO) for 30 min at room temperature. Cells were washed three times in RPMI, resuspended at $1x10^6$ cells/ml in complete medium, and intracellular free calcium concentration was monitored with a flow cytometer. After 3 min at 37°C, IIA1.6 cells were stimulated with 45 μg/ml $R_{ab}AM$ IgG or 30 μg/ml $R_{ab}AM$ $F(ab')_2$, and $[Ca^{2+}]_i$ was measured. The mean $[Ca^{2+}]_i$ was evaluated with the software FCS assistant 1.2.9 beta (Becton Dickinson).

Western blot analysis. Material was boiled in Sample Buffer, fractionated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Membranes were saturated with either 5% BSA or 5% skimmed milk (Régilait, Saint-Martin-Belle-Roche, France) diluted in Western Buffer (150 mM NaCl, 10 mM Tris and 0.5% Tween 20 (Merk, Schuchardt, Germany) pH 7.4), and incubated with the indicated antibodies followed by HRP-GAR or HRP-GAM. Labeled antibodies were detected using an ECL kit (Amersham Pharmacia biotech, Little Chalfont, Buckinghamshire).

Whole cell lysate analysis. IIA1.6 transfectants were stimulated at 37°C for indicated times with 30 μg/ml intact or 20 μg/ml $F(ab')_2$ fragments of $R_{at}AM$ IgG, and lysed by 3 cycles of

incubation for 1 min in liquid nitrogen followed by 1 min at 37°C in Lysis Buffer pH 8.0 (50 mM Tris pH8, 150 mM NaCl, 1% Tx100, 1 mM Na_3VO_4, 5 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1mM PMSF). Proteins were quantitated using a Biorad protein assay (Hercules, CA) and 40 μg of proteins were treated as described in Western blot analysis.

Immunoprecipitation. IIA1.6 transfectants were stimulated at 37°C for 3 min with 30 μg/ml intact or 20 μg/ml $F(ab')_2$ fragments of $R_{at}AM$ or $R_{ab}AM$, or treated with 100 mM pervanadate. Pervanadate was generated by mixing 1 ml of 20 mM Na_3VO_4 with 330 μl of 30% H_2O_2 followed by a 5-min incubation at room temperature, yielding a solution of 6 mM pervanadate. DT40 transfectants were stimulated at 37°C for 0.5, 1 or 3 min with 30 μg/ml $R_{ab}AC$ IgG. Cells were lysed in Lysis Buffer pH 7.4 (10 mM Tris pH 7.4, 150 mM NaCl, 1% Tx100, 1 mM Na_3VO_4, 5 mM NaF, 5 mM Sodium pyrophosphate, 0.4 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and 1 mM PMSF). Postnuclear lysates were immunoprecipitated with 2.4G2-coated sepharose beads, or with protein A-Sepharose beads (Amersham) coupled to rabbit anti-Grb2 or anti-Grap antibodies. Eluates from immunoadsorbents were treated as described in Western blot analysis.

GST fusion proteins. cDNA encoding the SH2 domain of SHIP1 was amplified by PCR, using as a template cDNA generated from RNA extracted from RBL-2H3 cells. cDNA encoding Grap-containing GST fusion protein was a gift from Dr. S. E. Shoelson (Joslin Diabetes Center, Boston, MA). GST-SHIP1 SH2 and GST-Grap cDNAs were inserted in pGEX-4T-2 (Amersham) and transfected into DH5-α *E. coli*. Bacteria producing GST-Grb2 SH2 were a gift from Dr. I. Broutin (UMR 8015 CNRS, Paris, France). Bacteria producing GST-Grb2 were a gift from Dr. S. Latour (INSERM U429, Paris, France). All fusion proteins

were produced in DH5-α *E. coli* following IPTG induction, purified on glutathione agarose (Sigma) and analyzed by SDS-PAGE. Soluble fusion proteins were eluted from glutathione agarose beads with a solution of 50 mM Tris, 25mM glutathione pH 8.0. The fusion proteins used in Fig. 3C were purchased from Santa Cruz Biotechnology. GST fusion proteins beads were incubated for 2 h in lysates from $1x10^7$ cells. Eluates from beads were treated as described in Western blot analysis.

Peptides and *in vitro* binding of proteins. Biotinylated peptides corresponding to the FcγRIIB ITIM, phosphorylated (pITIM) or not (ITIM), and to the FcγRIIB 16 C-terminal aminoacids, phosphorylated (pC-ter) or not (C-ter) were purchased from Sigma-Genosys (The Woodlands, Texas). They were coupled to streptavidin-agarose beads. Beads were incubated for 2 h with lysates from $1x10^7$ IIA1.6 cells or $2x10^7$ DT40 cells, or with soluble GST fusion proteins. Eluates from beads were treated as described in Western blot analysis.

Results

1. The ITIM is necessary, but not sufficient, for FcγRIIB to recruit SHIP1.

Because we reported previously that a deletion of the 16 C-terminal aminoacids of the FcγRIIB1 intracytoplasmic domain (FcγRIIB1 Δ314) abolished the coprecipitation of SHIP1 with the receptor (29), and because the deleted sequence contained a tyrosine residue, we examined the respective contributions of the ITIM tyrosine and of the C-terminal tyrosine of FcγRIIB in the recruitment of SHIP1. FcγRIIB1 bearing a point mutation of either the ITIM tyrosine (FcγRIIB1 Y309G) or of the C-terminal tyrosine (FcγRIIB1 Y326F) were stably expressed in the FcγR-negative variant of the murine B lymphoma A20/2J, IIA1.6. IIA1.6 transfectants expressing wild-type (wt) FcγRIIB1 were used as positive controls (Fig. 1A). Wt

and mutant FcγRIIB1 were coaggregated with BCR using intact Rabbit anti-Mouse Ig (R_{ab}AM) IgG antibodies that can bind both to BCR *via* their Fab portions and to FcγRIIB1 *via* their Fc portion. FcγRIIB1 were immunoprecipitated, and immunoprecipitates were Western blotted with anti-FcγRIIB, anti-phosphotyrosine and anti-SHIP1 antibodies. Wt and mutant FcγRIIB1 became tyrosyl-phosphorylated following coaggregation with BCR. Compared to wt FcγRIIB1, FcγRIIB1 mutants were less phosphorylated. The coprecipitation of SHIP1 with phosphorylated wt FcγRIIB1 was lost not only in cells expressing FcγRIIB1 Y309G, as expected, but also in cells expressing FcγRIIB1 Y326F, although the ITIM remained intact in this mutant (Fig. 1B). As observed previously (29), the coprecipitation of SHIP1 and SHIP2, was also lost in cells expressing FcγRIIB1 Δ314 (Fig. S1).

This loss of a detectable coprecipitation of SHIP1 with FcγRIIB1 Y326F was correlated with a loss of inhibition of Erk activation. Erk phosphorylation, induced upon BCR aggregation, was indeed decreased upon coaggregation of BCR with wt FcγRIIB1, but not upon coaggregation of BCR with either FcγRIIB1 Y309G or FcγRIIB1 Y326F (Fig. 2A).

Akt phosphorylation induced upon BCR aggregation, that was abolished upon coaggregation of BCR with wt FcγRIIB1, was partially inhibited upon coaggregation of BCR with FcγRIIB1 Y309G, FcγRIIB1 Y326F (Fig. 2B), or FcγRIIB1 Δ314 (Fig. S2A). In order to understand how FcγRIIB1 mutants could still inhibit Akt activation to some extent, we analyzed the colocalization of SHIP1 with wt or mutant FcγRIIB1 by confocal microscopy (Fig. S2B and C). As previously observed (36), SHIP1 colocalized with BCR-wt FcγRIIB1 coaggregates in more than 80% cells. The colocalization of SHIP1 with either BCR-FcγRIIB1 Δ314 or BCR-FcγRIIB1 Y309G coaggregates was reduced, but it could still be observed in 40-50% of the cells.

Altogether, these data indicate that the tyrosine contained in the C-terminal sequence of FcγRIIB1 contributes to the recruitment of SHIP1 and to FcγRIIB-dependent inhibition of Erk and Akt activation.

2. FcγRIIB contain a second tyrosine-based motif that binds the adapters Grb2/Grap *in vitro* and recruit these adapters *in vivo*.

The C-terminal tyrosine of FcγRIIB is within a consensus Grb2-binding site. Indeed, phosphorylated peptides corresponding to the 16 C-terminal aminoacids of FcγRIIB that were deleted in FcγRIIB1 Δ314 (pC-ter), but not the same non phosphorylated peptides (C-ter), precipitated Grb2 and Grap from a IIA1.6 cell lysate, but not the related adapters Nck, Nckβ or CrkL, that were all present in the lysate (Fig. 3A). pC-ter, but not C-ter, bound to GST fusion proteins containing Grb2 or Grap (Fig. 3B). Finally, pC-ter, but not C-ter, also bound to a GST fusion protein containing the SH2 domain of Grb2. This GST-Grb2 SH2 fusion protein failed to bind to a phosphorylated peptide corresponding to the FcγRIIB ITIM (pITIM). Conversely, a GST fusion protein containing the SH2 domain of SHIP1 bound to pITIM, but not to pC-ter (Fig. 3C). These data indicate that pC-ter can bind to the adapters Grb2 and Grap, but not to SHIP1. *In vitro* binding results from a direct interaction of pC-ter with the two adapters and, at least for Grb2, this interaction is *via* its SH2 domain. Conversely, pITIM can bind to SHIP1 (and SHIP2 (14)), but not to adapter molecules.

Based on the above *in vitro* results, we investigated whether adapter molecules would coprecipitate with phosphorylated FcγRIIB in IIA1.6 cells. FcγRIIB1 phosphorylation was induced either by coaggregating the receptors with BCR using R_{at}AM IgG antibodies or by treating cells with pervanadate. The coprecipitation of SHIP1 varied with the intensity of FcγRIIB1 phosphorylation. Neither Grb2 nor Grap coprecipitated with FcγRIIB1 in untreated cells. Grb2, but not Grap, detectably coprecipitated with FcγRIIB1 following coaggregation

with BCR. Both Grb2 and Grap coprecipitated with FcγRIIB1 following pervanadate treatment (Fig. 3D). Phosphorylated FcγRIIB1 therefore recruit the adapters Grb2 and Grap *in vivo*.

3. Grb2 and Grap interact with SHIP1/2 *in vitro* and *in vivo*.

Although pC-ter and pITIM bound specifically to the SH2 domains of Grb2 and SHIP1, respectively (Fig. 3C), pITIM precipitated not only SHIP1, but also Grb2, when incubated with IIA1.6 cell lysate (Fig. 4A). Grb2 was previously reported to bind SHIP1 (18), but not SHIP2, via its C-terminal SH3 domain (37). However, GST-Grb2 precipitated both SHIP1 and SHIP2 from IIA1.6 cell lysate (Fig. 4B). We therefore analyzed the binding of SHIP1 and SHIP2 to the three domains of Grb2 (SH3-N, SH2, SH3-C) separately. Neither GST-SH2 nor GST-SH3-N detectably precipitated SHIP1 or SHIP2 (although GST-SH3-N precipitated Sos), whereas GST-SH3-C precipitated SHIP1 and SHIP2, as did GST-Grb2 (Fig. 4B). The *in vitro* interactions of Grb2 with both SHIP1 and SHIP2 are therefore mediated by the C-terminal SH3 domain of Grb2.

In order to confirm these *in vitro* data, we examined the coprecipitation of SHIP1/2 with adapter proteins in IIA1.6 cells expressing wt FcγRIIB1. As detected by Western blotting with corresponding antibodies, Grb2, but not Grap, was precipitated by anti-Grb2 antibodies whereas Grap, but not Grb2, was precipitated by anti-Grap antibodies, and comparable amounts of each adapter were precipitated in all conditions. Small amounts of SHIP1 and SHIP2 coprecipitated with Grb2 in unstimulated cells. Higher amounts of both phosphatases coprecipitated with Grb2 following BCR aggregation and even higher amounts following the coaggregation of BCR with FcγRIIB1. Neither SHIP1 nor SHIP2 detectably coprecipitated with Grap in unstimulated cells. Minute amounts of SHIP2 coprecipitated with Grap following BCR aggregation and both SHIP1 and SHIP2 coprecipitated with Grap following

the coaggregation of BCR with FcγRIIB1 (Fig. 4C).

Taken together, these results indicate that the two known SH2 domain-containing inositol 5'-phosphatases SHIP1 and SHIP2 can bind *in vitro* to Grb2 and can associate *in vivo* with Grb2 and Grap in B cells.

4. Two tyrosine-based motifs are required for FcγRIIB to recruit either SHIP1 or Grb2.

To determine the respective contributions of the two FcγRIIB1 motifs in the binding of adapter-phosphatase complexes, we constructed an *in vitro* model of the intracytoplasmic domain of FcγRIIB. The C-ter peptide, phosphorylated or not, and the ITIM peptide, phosphorylated or not, were mixed in variable proportions and a constant amount of the mixture was used to coat agarose beads. These were used to precipitate SHIP1 and Grb2 from IIA1.6 cell lysate (Fig. 5A). pITIM alone, but not ITIM, precipitated SHIP1 and a small amount of Grb2. Conversely, pC-ter alone, but not C-ter, precipitated Grb2 and a small amount of SHIP1. The amount of SHIP1 precipitated by pITIM-coated beads decreased when beads were coated with decreasing amounts of pITIM and increasing amounts of C-ter (left panel), but not when beads were coated with decreasing amounts of pITIM and increasing amounts of pC-ter (right panel). Likewise, the amount of Grb2 precipitated by pC-ter-coated beads decreased when beads were coated with decreasing amounts of pC-ter and increasing amounts of ITIM (middle panel), but it increased when beads were coated with decreasing amounts of pC-ter and increasing amounts of pITIM (right panel). These results indicate that, when present on the same beads, pC-ter could enhance the *in vitro* binding of SHIP1 to pITIM and that, conversely, pITIM could enhance the *in vitro* binding of Grb2 to pC-ter.

In order to validate these *in vitro* observations *in vivo*, we examined whether the FcγRIIB ITIM contributes to the recruitment of Grb2, as the FcγRIIB C-terminal motif does for the recruitment of SHIP1, following the coaggregation of FcγRIIB1 with BCR in IIA1.6

transfectants. Both Grb2 and SHIP1 failed to coprecipitate not only with phosphorylated FcγRIIB1 Y326F, but also with phosphorylated FcγRIIB1 Y309G (Fig. 5B). Both the ITIM and the C-terminal motifs are therefore necessary for FcγRIIB to cooperatively recruit SHIP1 and Grb2, as a phosphatase-adapter complex.

5. Grb2 or Grap is required for FcγRIIB to recruit SHIP.

To investigate the respective roles of the two adapters Grb2 and Grap in this cooperative binding, we used the same *in vitro* model as in Fig. 5A with cell lysates from the chicken B cells DT40. These were wt cells, Grb2-deficient cells, Grap-deficient cells or Grb2- and Grap-deficient cells (30). Beads coated with pITIM and pC-ter required lower amounts of pITIM to precipitate SHIP from wt DT40 cell lysate (Fig. 6, right panel) than beads coated with pITIM and C-ter (Fig. 6, left panel). The same was observed in lysate from Grb2-deficient cells and in lysate from Grap-deficient cells, but not in lysate from Grb2- and Grap-deficient cells where comparable amounts of SHIP were precipitated by beads coated with pITIM and pC-ter or with pITIM and C-ter (Fig. 6). Either Grap or Grb2 is therefore necessary and sufficient to support the binding of SHIP to the FcγRIIB ITIM.

To confirm these *in vitro* data, wt and the three deficient DT40 cells were stably transfected with wt FcγRIIB1 (Fig. 7A). FcγRIIB1 were coagregated with the DT40 BCR by Rabbit anti-Chicken Ig ($R_{ab}AC$) IgG antibodies. In wt DT40 cells, SHIP coprecipitated with FcγRIIB1 upon coaggregation with BCR. Coprecipitation was lost in Grb2/Grap-doubly deficient cells (Fig. 7B). Coprecipitation was retained in Grb2- or Grap-single deficient cells, albeit in lower amount than in wt cells (Fig. 7C). One adapter is therefore necessary and sufficient for FcγRIIB1 to recruit SHIP but both are required for an optimal recruitment.

Discussion

We show here 1) that, in addition to the ITIM, the intracytoplasmic domain of FcγRIIB contains a second tyrosine-based motif that recruits the SH2 domain-containing adapters Grb2 and Grap, 2) that these adapters interact with the inositol-phosphatases SHIP1 and SHIP2 *via* their C-terminal SH3 domain, 3) that the two tyrosine-based motifs each contribute to the recruitment of both SHIP1 and Grb2 by FcγRIIB, and 4) that adapters are necessary for FcγRIIB to recruit SHIP1.

FcγRIIB-dependent negative regulation is thought to depend on the phosphorylated ITIM. The FcγRIIB ITIM was shown to be necessary on the basis of mutational analyses. The deletion of a 13-aminoacid sequence containing the ITIM (4) or the point mutation of the ITIM tyrosine (5) was indeed sufficient to abrogate most of the inhibitory properties of FcγRIIB1 in B cells, T cells and mast cells. The FcγRIIB ITIM was concluded to be sufficient for inhibition on the basis of a study showing that a chimeric molecule whose intracytoplasmic domain was constituted by residues 53-68 (VKFSRSAEPPAYQQGQ) of the human TCRζ subunit and residues 303-315 (AENT**ITYSLL**KHP) containing the ITIM (in bold type) of murine FcγRIIB, linked by a two-serine spacer, could inhibit BCR-induced calcium mobilization and IL-2 secretion in IIA1.6 cells (28). Inhibition of BCR-mediated IL-2 secretion by this chimera was, however, half that induced by wt FcγRIIB1, and the authors suggested that other sequences, in FcγRIIB1, might be required to maximize inhibition. One also notices that a TCRζ tyrosine residue was present in the construction, in addition to the ITIM tyrosine, as well as another two prolines and four serines, that could potentially recruit cytosolic molecules. The conclusion that the FcγRIIB ITIM is sufficient to account for the inhibitory properties of the receptor may therefore not be as firmly established than it is usually accepted.

The inhibitory properties of FcγRIIB could be accounted for by the ability of the receptor to recruit SHIP1 (23-25). A role of SHIP2 was also suggested in FcγRIIB-dependent

negative regulation of LPS-activated B cells (27). We show here that cytosolic molecules other than the two phosphatases are recruited by phosphorylated FcγRIIB1. These are the two adapter molecules Grb2 and Grap, that bind to the C-terminal motif *via* their SH2 domain. The intracytoplasmic domain of FcγRIIB therefore contains two tyrosine-based motifs that bind specifically the SH2 domain of SHIP1 and the SH2 domain of Grb2 respectively. The recruitment of phosphatases, however, required an intact adapter-binding motif and, conversely, the recruitment of adapters required an intact phosphatase-binding motif. These observations could be explained by a cooperative binding of phosphatases and adapters to FcγRIIB1. Supporting this possibility, we found that Grb2 could interact with SHIP1 and SHIP2 *via* its C-terminal SH3 domain, and that SHIP1 (38) and SHIP2 coprecipitated with Grb2 and Grap in IIA1.6 cells. Coprecipitation of phosphatases with adapters was enhanced following BCR aggregation and further enhanced following coaggregation of BCR with FcγRIIB1. Since the interactions between SH3 domains and proline-rich sequences are not inducible *per se*, this suggests that phosphotyrosine-dependent interactions may stabilize phosphotyrosine-independent interactions when adapters and phosphatases are brought in proximity within signaling complexes. Conversely, phosphotyrosine-independent interactions may stabilize phosphotyrosine-dependent interactions. Using a model of the FcγRIIB1 intracytoplasmic domain, in which peptides containing the two SH2 domain-binding sites were bound to the same beads, we indeed found that, when phosphorylated, the C-terminal peptide enhanced the binding of SHIP1 to the phosphorylated ITIM peptide, and that conversely, when phosphorylated, the ITIM peptide enhanced the binding of Grb2 to the phosphorylated C-teminal peptide. This reciprocal enhancement of phosphatase and adapter binding suggests that the recruitment of SHIP1 and Grb2 by FcγRIIB1 involves cooperative binding within a trimolecular complex composed of the phosphorylated receptor, the phosphatase and the adapter.

This conclusion may not be restricted to the interactions of FcγRIIB1, SHIP1 and Grb2. Indeed, molecules that contain two SH2 domains require the cooperative binding of these two domains to two sequences containing phosphorylated tyrosines in order to be recruited *in vivo*. Thus, the recruitment of the protein tyrosine kinases ZAP-70 and Syk (39,40), or of the tyrosine phosphatase SHP-1 (41), requires the conservation of their two SH2 domains and the conservation of the two tyrosines of ITAMs in immunoreceptors (42) or of the two ITIMs in Killer cell Inhibitory Receptors (33,43) respectively. Moreover, molecules that contain a single SH2 domain were found to require the cooperation of other SH2 domain-containing molecules in order to be recruited (44). We wish therefore to propose that one SH2 domain alone may not be sufficient to enable stable interactions between signaling molecules.

Stable interactions between FcγRIIB1 and SHIP1 can be operationally defined as enabling the coprecipitation of the phosphatase with the receptor. Based on our results, such an interaction would require the two SH2-binding motifs in FcγRIIB1 and adapter molecules. SHIP1/2-Grb2/Grap complexes would indeed bind to FcγRIIB1 with a high avidity resulting from the combined affinities of the SHIP1/2 SH2 domain for the ITIM and of the Grb2/Grap SH2 domain for the C-terminal motif. A stable interaction between FcγRIIB1 and SHIP1 correlates with an optimal inhibition of B cell responses. However, FcγRIIB1 Y309G and FcγRIIB1 Δ314 retained some ability to inhibit Akt phosphorylation, although they failed to coprecipitate SHIP1. This inhibition could be explained by an unstable recruitment of SHIP1, directly to the ITIM of mutant FcγRIIB1 Δ314, or indirectly to the C-terminal motif of FcγRIIB1 Y309G *via* adapters. This is consistent with the partial colocalization of SHIP1 with mutant receptors observed in IIA1.6 cells. Whatever the mechanism of unstable interactions, adapters may stabilize the recruitment of single SH2 domain-containing phosphatases by FcγRIIB, and thus modulate FcγRIIB signaling.

Grb2 and Grap being coexpressed in B cells (45), we examined their respective roles

with Grb2- and/or Grap-deficient DT40 cells. Using the same *in vitro* model of FcγRIIB1 intracytoplasmic domain, we found a similar enhancement of the binding of SHIP1 to the phosphorylated ITIM peptide by the phosphorylated C-terminal peptide in cell lysates from wt DT40 cells as from IIA1.6 cells. This enhancement was abolished in Grb2/Grap doubly-deficient cells. Importantly, the same was observed *in vivo* since SHIP coprecipited with FcγRIIB1 in wt DT40 cells, but not in Grb2/Grap doubly-deficient DT40 cells. These experiments thus provide a genetic evidence that adapters are necessary for FcγRIIB to recruit SHIP. Noticeably, Grb2 and Grap could substitute for each other : the phosphorylated C-terminal peptide enhanced the binding of SHIP to the phosphorylated ITIM peptide and some SHIP coprecipitated with FcγRIIB1 in Grb2-deficient cells and in Grap-deficient cells. Grb2 and Grap were previously described to replace each other in T cells where they couple the Hematopoietic Progenitor Kinase-1 to phosphorylated proteins such as the Linker of Activation of T cells (30). Although SHIP1 could be recruited by FcγRIIB1 in the presence of either Grb2 or Grap, the coprecipitation of SHIP with FcγRIIB1 was of a lower magnitude in single deficient DT40 cells than in wt DT40 cells. Both adapters may therefore be required for an optimal *in vivo* recruitment of SHIP1 by FcγRIIB.

Grb2 and Grap being composed of one SH2 domain and two SH3 domains, when they are recruited by FcγRIIB *via* their SH2 domain, their C-terminal SH3 domain is engaged with SHIP1/2 but their N-terminal SH3 domain remains free to bind other proline-rich molecules. These could either be sequestered from nearby signaling complexes or/and contribute to FcγRIIB-derived signals (46). Grb2 associates with a variety of molecules *via* its N-terminal SH3 domain and, interestingly, Grap associates with only some molecules among Grb2 partners (45). It was recently reported that Ras-dependent T cell proliferation and IL-2 production were enhanced in Grap-deficient mice (47), suggesting that Grap itself could mediate negative regulation. When recruited by FcγRIIB, adapters may thus reinforce

inhibition. Finally, our work provides evidence that FcγRIIB may have a more complex function than simply recruiting SHIP. They indeed appear to function as scaffold proteins that modulate the composition of signaling complexes generated by immunoreceptors with which they are coengaged.

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Abbreviations

Ig, Immunoglobulin; FcR, Fc Receptor; BCR, B Cell Receptor; TCR, T Cell Receptor; ITAM, Immunoreceptor Tyrosine-based Activation Motif; ITIM, Immunoreceptor Tyrosine-based Inhibition Motif; SHIP, SH2 domain-containing Inositol 5'-Phosphatase; PI(3,4,5)P3, PhosphatidylInositol-(3,4,5)-triPhosphate; PLCγ, PhosphoLipase C-γ; R_{at}AM, Rat Anti Mouse Ig; R_{ab}AM, Rabbit Anti Mouse Ig; R_{ab}AC, Rabbit Anti Chicken Ig; MAR_{at}, Mouse Anti Rabbit Ig; GAR_{ab}, Goat Anti Rabbit Ig; GST, Glutathione S-Transferase; IL-2, Interleukin-2.

Figure Legends

Figure 1. The C-terminal tyrosine (Y326) of the intracytoplasmic domain of FcγRIIB1 is mandatory for SHIP1 coprecipitation. (A) *Schematic representation of FcγRIIB mutants and peptides used in this study*. Aminoacid sequence of the ITIM and C-ter peptides are indicated in black and white, respectively. Histograms show the expression of wt and mutated FcγRIIB1 assessed by indirect immunofluorescence. Bold histograms: 2.4G2 and FITC-MAR F(ab')$_2$; thin histograms: FITC-MAR F(ab')$_2$ only. **(B)** *Coprecipitation of SHIP1 with wt and mutated FcγRIIB1 in IIA1.6 transfectants*. Cells were stimulated (+) or not (-) with R_{ab}AM IgG for 3 min. Cells were lysed and FcγRIIB1 were precipitated with 2.4G2. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr and anti-SHIP1 antibodies.

Figure 2. The C-terminal tyrosine (Y326) of the intracytoplasmic domain of FcγRIIB1 is mandatory for abrogation of Erk phosphorylation, and complete inhibition of Akt phosphorylation. (A) *Erk phosphorylation following coaggregation of BCR with wt and mutated FcγRIIB in IIA1.6 transfectants*. Cells were unstimulated (-) or stimulated (+) with either R_{at}AM F(ab')$_2$ or R_{at}AM IgG for the indicated periods of time. Cells were lysed, and proteins were fractionated by SDS-PAGE and Western blotted with anti-pErk or anti-Erk antibodies. **(B)** *Akt phosphorylation following coaggregation of BCR with wt and mutated FcγRIIB in IIA1.6 transfectants*. Cells were unstimulated (-) or stimulated (+) with either R_{at}AM F(ab')$_2$ or R_{at}AM IgG for the indicated periods of time. Cells were lysed, and proteins were fractionated by SDS-PAGE and Western blotted with anti-pAkt or anti-Akt antibodies.

Figure 3. The 16 C-terminal aminoacids of the intracytoplasmic domain of FcγRIIB1 bind *in vitro* to Grb2 and Grap that coprecipitate with phosphorylated FcγRIIB1 *in vivo*. **(A)** In vitro *binding of adapters to FcγRIIB C-ter peptides*. Agarose beads coated with nonphosphorylated (C-ter) or phosphorylated (pC-ter) peptides corresponding to the 16 C-terminal aminoacids of FcγRIIB, were incubated with IIA1.6 cell lysate. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1, anti-Nckβ, anti-Nck, anti-CrkL, anti-Grap and anti-Grb2 antibodies. Whole cell lysate (WCL) was used as a positive control. **(B)** In vitro *binding of adapter-containing GST fusion proteins to FcγRIIB C-ter peptides*. Agarose beads coated with pC-ter or C-ter were incubated with GST-Grap or GST-Grb2. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-GST antibodies. **(C)** In vitro *binding of SH2 domain-containing GST fusion proteins to FcγRIIB C-ter and ITIM peptides*. Agarose beads coated with nonphosphorylated or phosphorylated ITIM or C-ter peptides were incubated with GST-SHIP1 SH2 or GST-Grb2 SH2. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-GST antibodies. **(D)** *Coprecipitation of adapters with wt FcγRIIB1 in IIA1.6 transfectants*. Cells were stimulated (+) or not (-) with R_{at}AM IgG or treated with pervanadate for 3 min. Cells were lysed and FcγRIIB were precipitated with 2.4G2. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr, anti-SHIP1, anti-Grb2 and anti-Grap antibodies.

Figure 4. Grb2 and Grap bind *in vitro* and *in vivo* to SHIP1/2. **(A)** In vitro *binding of SHIP1 and Grb2 to FcγRIIB C-ter and ITIM peptides*. Agarose beads coated with nonphosphorylated or phosphorylated peptides corresponding to the FcγRIIB ITIM or C-ter, were incubated with IIA1.6 cell lysate. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1 and anti-Grb2 antibodies. **(B)** In vitro *binding of*

SHIP1/2 to fusion proteins containing Grb2 or its domains. Agarose beads coated with GST, GST-Grb2, GST-Grb2 SH3N, GST-Grb2 SH2 or GST-Grb2 SH3C, were incubated with IIA1.6 cell lysate. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-SHIP2, anti-SHIP1 and anti-Sos antibodies. **(C)** *Coprecipitation of SHIP1/2 with Grb2 and Grap in IIA1.6 FcγRIIB1 transfectants*. Cells were unstimulated (-) or stimulated (+) with either R_{at}AM F(ab')$_2$ or R_{at}AM IgG for 3 min. Cells were lysed, and Grb2 and Grap were precipitated with anti-Grb2 or anti-Grap antibodies respectively. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-Grb2, anti-Grap, anti-SHIP2 and anti-SHIP1 antibodies. Whole cell lysate (WCL) was used as a positive control.

Figure 5. The two tyrosine-based motifs cooperate to bind SHIP1 and Grb2 *in vitro* and are required for FcγRIIB to recruit either SHIP1 or Grb2 *in vivo*. **(A)** In vitro *binding of SHIP1 and Grb2 to beads coated with a mixture of FcγRIIB C-ter and ITIM peptides*. Four μg of nonphosphorylated or phosphorylated FcγRIIB C-ter and ITIM peptides, were mixed in variable proportions and bound to agarose beads. Beads were incubated in IIA1.6 cell lysate. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1 and anti-Grb2 antibodies. **(B)** *Coprecipitation of Grb2 with wt and mutated FcγRIIB1 in IIA1.6 transfectants*. Cells were stimulated (+) or not (-) with R_{ab}AM IgG for 3 min. Cells were lysed and FcγRIIB were precipitated with 2.4G2. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr, anti-SHIP1, and anti-Grb2 antibodies.

Figure 6. Adapters are required for stabilizing the *in vitro* binding of SHIP to the FcγRIIB pITIM. Twelve μg of nonphosphorylated or phosphorylated peptides corresponding to the FcγRIIB C-ter and ITIM were mixed in variable proportions and bound to agarose

beads. Beads were incubated in cell lysates from wt, Grb2$^{-/-}$Grap$^{+/+}$, Grb2$^{+/+}$Grap$^{-/-}$ or Grb2$^{-/-}$ Grap$^{-/-}$ DT40 cells. Precipitated material was fractionated by SDS-PAGE and Western blotted with anti-SHIP1 antibodies.

Figure 7. Adapters are mandatory for FcγRIIB1 to recruit SHIP *in vivo*. (A) *Expression of BCR and FcγRIIB1 in DT40 transfectants*. Histograms show the expression of BCR or FcγRIIB1 in DT40 cells, assessed by indirect immunofluorescence. Upper panel, bold histograms: R_{ab}AC and FITC-GAR_{ab} F(ab')$_2$; thin histograms: FITC- GAR_{ab} F(ab')$_2$ only. Lower panel, bold histograms: 2.4G2 and FITC-MAR_{at} F(ab')$_2$; thin histograms: FITC-MAR_{at} F(ab')$_2$ only. **(B)** In vivo *recruitment of SHIP by FcγRIIB1 in wt or Grb2/Grap doubly-deficient DT40 transfectants*. Wt or Grb2$^{-/-}$Grap$^{-/-}$ DT40 cells were stimulated or not with R_{ab}AC IgG for the indicated periods of time (in min). Cells were lysed and FcγRIIB were precipitated with 2.4G2. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr and anti-SHIP1 antibodies. **(C)** In vivo *recruitment of SHIP by FcγRIIB1 in wt or deficient DT40 transfectants*. Wt, Grb2$^{-/-}$Grap$^{+/+}$, Grb2$^{+/+}$Grap$^{-/-}$ or Grb2$^{-/-}$Grap$^{-/-}$ DT40 cells were stimulated (+) or not (-) with R_{ab}AC IgG for 1 min. Cells were lysed and FcγRIIB were precipitated with 2.4G2. Immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-FcγRIIB, anti-pTyr and anti-SHIP1 antibodies.

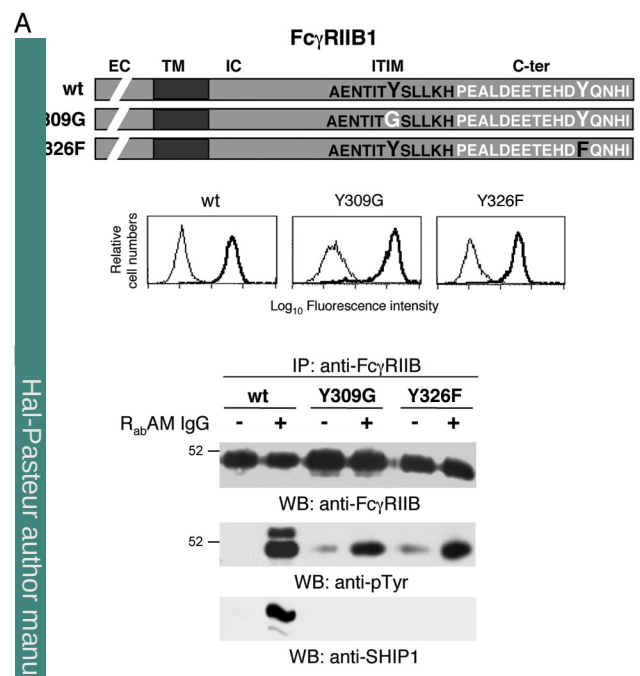

Figure 1

A

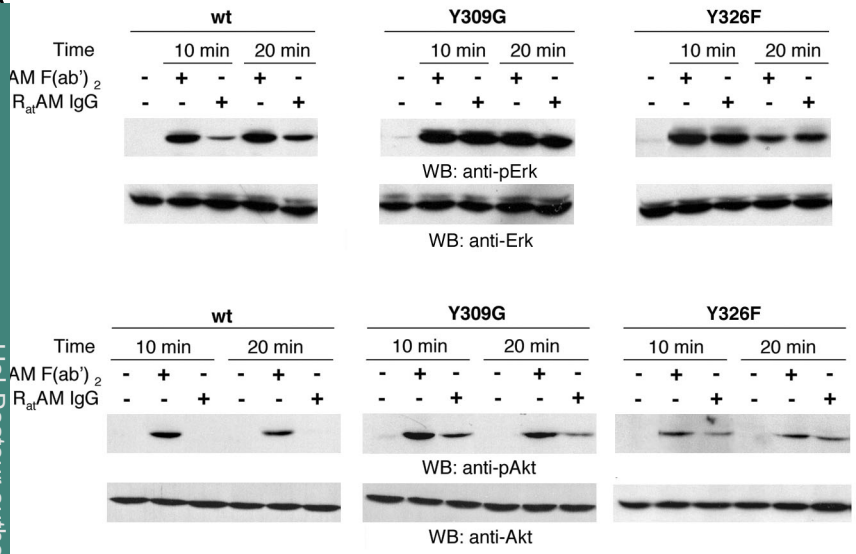

Figure 2

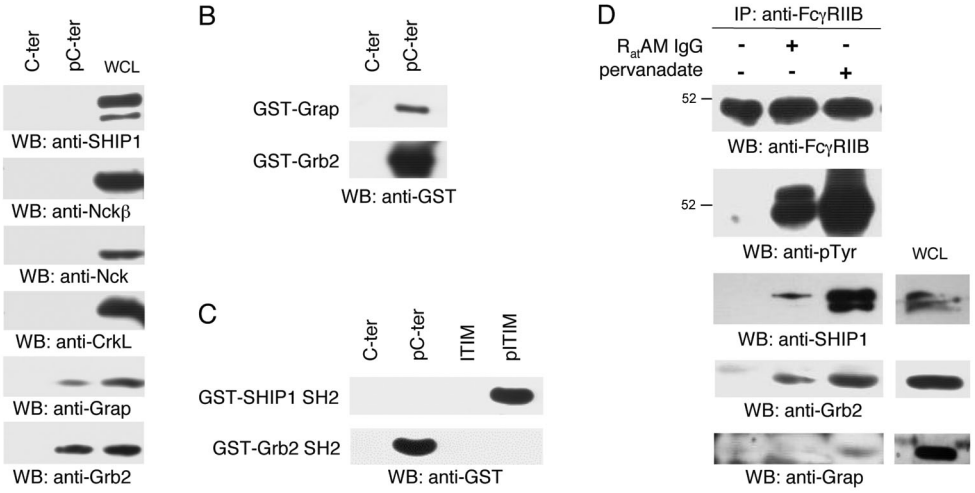

Figure 3

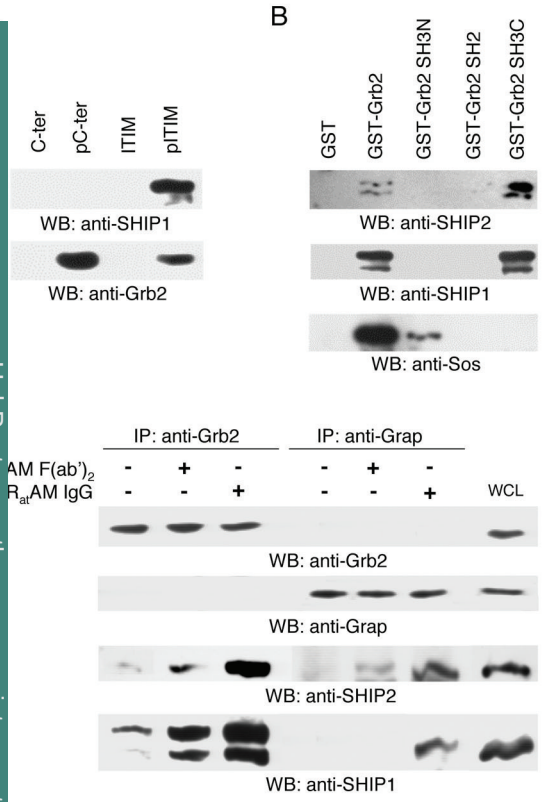

Figure 4

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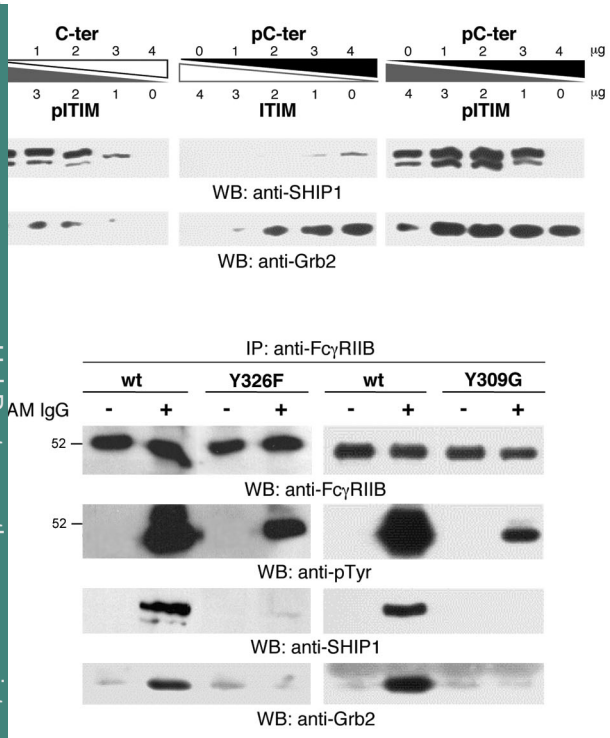

Figure 5

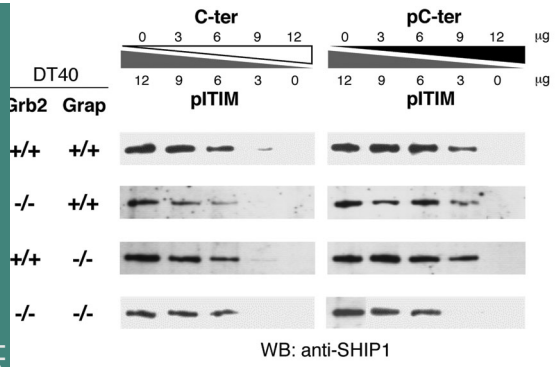

Figure 6

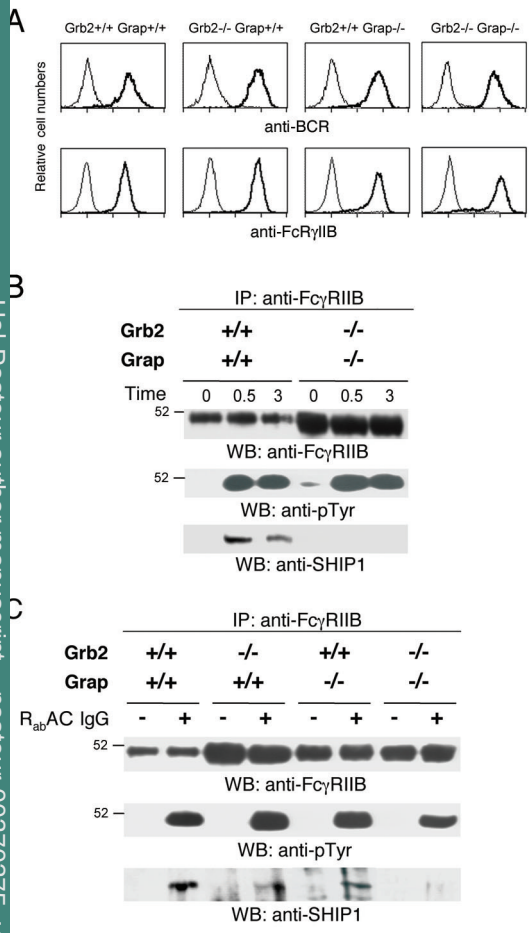

Figure 7