Src Homology 2 Domain-Containing Inositol Polyphosphate Phosphatase Regulates NF-κB-Mediated Gene Transcription by Phagocytic FcγRs in Human Myeloid Cells[1]

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FcγR-mediated phagocytosis is accompanied by the generation of tissue-damaging products such as inflammatory cytokines and reactive oxygen species. Hence, the phagocytic response must be a tightly regulated process. Recent studies have established that clustering FcγR on human myeloid cells causes tyrosine phosphorylation of Src homology 2 domain-containing inositol polyphosphate phosphatase (SHIP). However, it is not known how these immunoreceptor tyrosine-based activation motif (ITAM)-bearing phagocytic FcγR activate SHIP, or whether the activation of SHIP by ITAMs has any functional relevance. Experiments addressing the mechanism of SHIP association with ITAMs have been done in in vitro systems using phosphopeptides. In this study we undertook to dissect the molecular mechanism by which SHIP associates with the native ITAM-FcγR and becomes phosphorylated. In this report we provide evidence that first, SHIP is indeed phosphorylated by ITAM-FcγR, using cell systems that lack FcγRIIb expression; second, coimmunoprecipitation experiments demonstrate that SHIP associates with native ITAM-bearing FcγRIIa in vivo; and third, phosphorylation of SHIP by FcγRIIa is inhibited by overexpressing either the SHIP Src homology 2 domain or a dominant negative mutant of Shc. In contrast, SHIP phosphorylation was not inhibited by a dominant negative mutant of Grb2. We extend these observations to show that SHIP activation by ITAM-FcγR down-regulates NF-κB-induced gene transcription. These findings both provide a molecular mechanism for SHIP association with native ITAM-bearing receptors and demonstrate that SHIP association with ITAM-FcγR serves to regulate gene expression during the phagocytic process. ***The Journal of Immunology,*** **2002, 169: 4370–4378.**

Macrophages, playing an important role in the adaptive immune response, phagocytose infectious particles via FcγR and complement receptors (1). Because phagocytosis is often accompanied by the generation of tissue-damaging products such as reactive oxygen species and inflammatory cytokines, a tight regulation of this phagocytic process would seem mandatory. Although much is known about the molecular events leading to the activation process initiated by FcγR, it is not clear how this activation process is regulated.

Macrophages express three classes of FcγR: I, II, and III (2). Although the expression of FcγRI and III is common to both murine and human hematopoietic cells, the expression of FcγRII is different between the two species. Thus, while human cells express two functionally different forms of FcγRII (i.e., IIa and IIb, products of two separate genes), mouse cells express FcγRIIb but not FcγRIIa, having no gene for FcγRIIa. FcγRI, III, and IIa are activating receptors associated with immunoreceptor tyrosine-based activation motifs (ITAM)[3] that are present either within the cytoplasmic tail of the receptor as in FcγRIIa (3) or within the associated low-m.w. FcRγ subunit (4). In contrast, FcγRIIb is an inhibitory receptor that has in its cytoplasmic tail an immunoreceptor tyrosine-based inhibition motif (ITIM). The extracellular domains of FcγRIIa and IIb are virtually identical, a fact that has complicated the detection and study of FcγRIIb in human macrophages. However, recent studies using novel reagents established conclusively the presence of FcγRIIb protein in human macrophages (5, 6). FcγRIIb serves to down-regulate phagocytosis of IgG-coated particles, as demonstrated in several studies (7, 8), using as its effector molecule Src homology (SH)2 domain-containing inositol polyphosphate phosphatase (SHIP). We and others have well characterized the molecular details of SHIP association with FcγRIIb (9–11).

SHIP, expressed exclusively in hematopoietic cells, is a multidomain cytosolic protein, which contains not only an inositol phosphatase domain but also several protein interaction domains (12, 13) including 1) a SH2 domain that associates with high affinity to the phosphorylated ITIM of FcγRIIb (9–11) and with phosphorylated Shc (13, 14); 2) a proline-rich domain that constitutively associates with the SH3 domain of the adapter protein Grb2 (13, 15); and 3) multiple sites of tyrosine phosphorylation that are involved in SHIP association with Shc (14, 16, 17). SHIP hydrolyzes phosphatidylinositol-3,4,5-trisphosphate, a product of phosphatidylinositol 3-kinase, which is required for the activation of several enzymes that contain pleckstrin homology domains, such as Bruton's tyrosine kinase (18), involved in intracellular calcium mobilization; Akt (19, 20), an enzyme involved in cell survival; and Vav (21), the guanine nucleotide exchange factor for the Rho family of GTPases. The role of SHIP as an inhibitory

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[3] Abbreviations used in this paper: ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibition motif; SH, Src homology; GAM, goat anti-mouse Ig; SHIP, SH2 domain-containing inositol polyphosphate phosphatase; Erk, extracellular signal-regulated kinase; BMM, bone marrow-derived macrophage.

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protein working via the ITIM-bearing FcγRIIb as well as through ITAM-bearing receptors is well established both in vitro using cell lines and in vivo using SHIP-deficient mice. Thus, the presence of SHIP attenuates B cell receptor-mediated activation and FcεRI-mediated activation when FcγRIIb is coclustered with these receptors (11, 15, 19, 20, 22–24). Likewise, Greenberg et al. (25) demonstrated that SHIP-deficient murine macrophages display enhanced phagocytic efficiency in comparison to the wild-type macrophages. Paradoxically, the inhibitory influence of SHIP is exerted even when activating receptors alone are clustered, without invoking FcγRIIb, as is elegantly established in genetically altered mice and in avian B cell lines (23, 24, 26, 27), indicating that SHIP serves as a modulator of activation events independently of its being recruited by FcγRIIb. Although the molecular mechanism of SHIP association with the ITIM is well explored, it is unclear how SHIP associates with ITAMs and becomes phosphorylated by ITAM-bearing receptors. Likewise, it is unclear whether the activation of SHIP by ITAM-FcγR has any functional consequence.

We and others (28, 29) earlier reported the activation and association of SHIP with ITAM-FcγR in human monocytes. In light of our current knowledge that FcγRIIb protein is present in human monocytes, we undertook to test whether the previously reported ITAM-induced SHIP activation could be explained by the presence of trace amounts of intact Ab in the $F(ab')_2$ fractions of our activating Abs that could have involved FcγRIIb coclustering. Therefore, we analyzed the ability of ITAM-FcγR to induce SHIP phosphorylation in the absence of any functional FcγRIIb. We report here, using COS-7 transfection models that lack endogenous SHIP and FcRs, and knockout mouse models that lack FcγRIIb expression, that the ITAM-FcγR are indeed capable of inducing SHIP phosphorylation. Coimmunoprecipitation experiments revealed an in vivo association of SHIP with FcγRIIa, an interaction that might occur either directly or via an adapter molecule. Using transfection systems in which dominant negative forms of the SHIP-associated adapters, Grb2 and Shc, were overexpressed we demonstrate that, while SHIP is capable of a direct interaction in vitro with the ITAM of FcγRIIa, the association of SHIP with FcγRIIa in vivo is indirect and requires the presence of Shc. We extend these studies to provide evidence that SHIP activation serves to down-regulate gene transcription induced by the engagement of phagocytic FcγR in human myeloid cells.

Materials and Methods

Cells, Abs, and reagents

THP-1 human monocytic cells and COS-7 cells were obtained from American Type Culture Collection (Manassas, VA). P388D1 mouse macrophage cells stably expressing human FcγRIIa were a kind gift from Dr. J. Edberg (University of Alabama, Birmingham, AL). P388D1 and COS-7 cells were maintained in DMEM supplemented with 10% FBS. THP-1 cells were maintained in RPMI supplemented with 10% FBS. $F(ab')_2$ of anti-FcγRI mAb 32.2 and Fab of anti-FcγRIIa Ab IV.3 were obtained from Medarex (Annandale, NJ). Rabbit polyclonal anti-Shc, anti-extracellular signal-regulated kinase (Erk), and anti-phosphotyrosine Ab 4G10 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-SHIP Ab was a generous gift from Dr. K. M. Coggeshall (Oklahoma Medical Research Foundation, Oklahoma City, OK). Anti-human CD32 and anti-mouse CD16/32 were purchased from BD PharMingen (San Diego, CA). The NF-κB-luciferase reporter plasmid was purchased from Stratagene (La Jolla, CA) and the plasmid pEGFP was purchased from Clontech Laboratories (Palo Alto, CA).

Culture of murine bone marrow-derived macrophages (BMM)

Strain-matched wild-type and FcγRII-deficient mice were purchased from Taconic Farms (Germantown, NY). BMM were derived as previously described (30). Briefly, bone marrow cells were cultured in RPMI containing 5% FBS and supplemented with 50 ng/ml CSF-1 for 5 days. The BMMs were dissociated from the plates with Cell Dissociation Buffer (Life Technologies, Rockville, MD) and activated by clustering FcγR with mAb 2.4G2 (BD PharMingen) followed by $F(ab')_2$ of goat anti-rat IgG secondary Ab (Pierce, Rockford IL).

Immunoprecipitation and Western blotting

Cell activation, immunoprecipitation, and Western blotting were performed as previously described (30). Briefly, THP-1 cells and transfected COS-7 cells were activated by clustering FcγRI and or FcγRIIa with mAb 32.2 and mAb IV.3 Fab followed by goat anti-mouse Ig (GAM) secondary Ab. Resting and activated cells were lysed in TN1 buffer (50 mM Tris (pH 8), 10 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1% Triton X-100, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin), and postnuclear lysates were incubated overnight with the Ab of interest and protein G-agarose beads (Life Technologies) or GAM covalently linked to Sepharose, depending on the Ab. Immune complexes bound to beads were washed in TN1 and boiled in SDS sample buffer (60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% 2-ME) for 5 min. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the Ab of interest and appropriate HRP-conjugated secondary Abs. The filters were then developed by ECL.

Immunoblot data quantitation

The ECL signal was quantitated using a scanner and a densitometry program (Scion Image; National Institutes of Health). To quantitate the phosphotyrosine signal in the activated samples, we first subtracted background, normalized the signal to the amount of precipitated protein, and plotted the values obtained by subtracting the value in unstimulated samples, as previously described (30).

Far Western assay

Far Western assays were performed as previously described (9). Briefly, immunoprecipitated proteins were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then probed with 1 μM biotinylated, doubly phosphorylated ITAM peptide from FcγRIIa (described in Ref. 9) or pITIM from FcγRIIb (described in Ref. 28) overnight at 4°C. The blots were subsequently probed with HRP-conjugated streptavidin (Pierce) and developed by ECL. Bands were quantitated as described above.

Transfection of COS-7 cells

COS-7 cells were transfected as previously described (30). Briefly, cells were grown on culture dishes until they were 60–70% confluent. Two micrograms of cDNA for FcγRI α-chain in pCEXV3 (kindly provided by Dr. J. Ravetch, Rockefeller University, New York, NY), 2 μg of γ subunit cDNA in pSVL (a gift from Dr. J.-P. Kinet, Harvard Medical School, Boston, MA), 1 μg of SHIP cDNA in pcDNA3, 10 μg each of SHIP SH2 domain (kindly provided by Dr. K. M. Coggeshall), and Shc Y-F and Grb2 SH3 (a kind gift from Dr. B. Mayer, Harvard Medical School) mutant were mixed in various combinations with Lipofectamine 2000 reagent (Life Technologies). The DNA mix was added to cells in serum-free DMEM and incubated for 3 h at 37°C in a CO_2 incubator. The medium was then replaced by DMEM supplemented with 10% FBS. The cells were harvested 48 h later and analyzed for expression of the transfected cDNAs by flow cytometry and Western blotting. Having ensured that the various transfectants expressed comparable levels of protein, we then examined SHIP phosphorylation in response to FcγR clustering.

Transfection of THP-1 cells and luciferase assays

For analysis of SHIP influence on NF-κB transcriptional activity, THP-1 cells were transfected by electroporation (310 V, 950 uF; Gene Pulser II; Bio-Rad, Hercules, CA) with 20 μg of wild-type SHIP or SHIP SH2 domain in pCDNA3, 5 μg of NF-κB-luc plasmid, and 2 μg of pEGFP to normalize for transfection efficiency. Transfectants were harvested 24 h later and activated by clustering FcγRI or FcγRIIa by methods described above for 6 h at 37°C. The cells were lysed in 100 μl of cell culture lysis reagent (Promega, Madison, WI). Luciferase activity was measured using the Promega luciferase assay reagent. Data are represented as graphs indicating the fold induction of NF-κB activity in cells activated by clustering FcRs over those that were not activated. Data points are expressed as mean and SD of three independent experiments. Statistical analysis was performed by Student's *t* test.

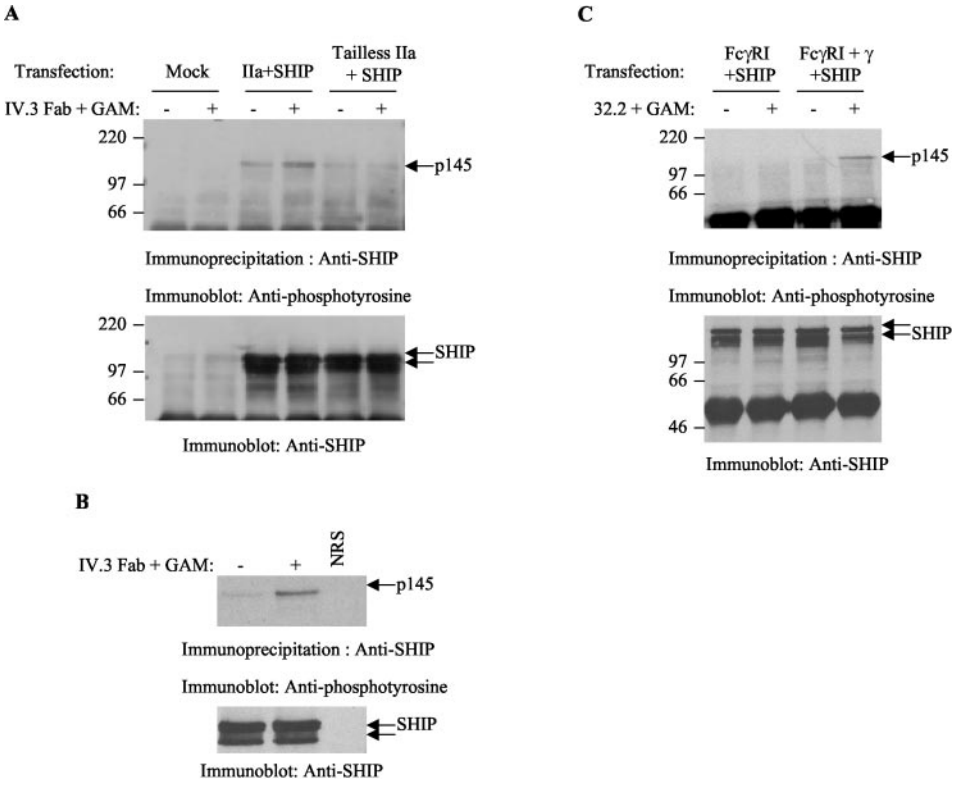

FIGURE 1. SHIP phosphorylation is induced by ITAM-FcγR in transfected cells. *A*, Transiently transfected COS-7 cells were activated by clustering FcγRIIa with IV.3 Fab and GAM for 3 min, followed by immunoprecipitation with anti-SHIP Ab. Tyrosine phosphorylation of SHIP was assessed by immunoblotting with anti-phosphotyrosine Ab (*upper panel*). The membrane was reprobed with anti-SHIP Ab to ensure equal loading (*lower panel*). *B*, P388D1 mouse macrophage cell line stably expressing FcγRIIa was activated by clustering FcγRIIa as indicated above, and SHIP phosphorylation was assessed. *C*, COS-7 cells were transiently transfected to express the proteins indicated. Cells were activated by clustering FcγRI with 32.2 $F(ab')_2$ and GAM, and phosphorylation of SHIP was assessed as above. These data are representative of three independent experiments. Molecular weights are indicated to the *left* of the panels.

Results

SHIP phosphorylation is induced by ITAM-FcγR in the absence of FcγRIIb

Our previous experiments indicated that clustering FcγRIIa with mAb IV.3 Fab and GAM or FcγRI with mAb 32.2 $F(ab')_2$ and GAM induced SHIP phosphorylation (28). Subsequent work from our laboratory and others established that human monocytes and the monocyte-like cell lines THP-1 and U937 express functional FcγRIIb (5, 6), raising the possibility that the SHIP phosphorylation observed in response to FcγRIIa or FcγRI clustering might be explained by the coligation of FcγRIIb by contaminating intact IgG in the anti-FcγR mAb preparations. Hence, we undertook to test whether ITAM-FcγR can induce tyrosine phosphorylation of SHIP in the complete absence of FcγRIIb. For this, we used three different approaches involving transiently transfected COS-7 cells, P388D1 mouse macrophage cells stably transfected to express human FcγRIIa. and BMMs from FcγRII-deficient mice.

First, to test whether FcγRIIa can induce SHIP phosphorylation in the absence of FcγRIIb, COS-7 cells, which lack endogenous expression of SHIP and FcRs, were transiently transfected to express a wild-type (Fig. 1*A*, *lanes 3* and *4*) or a tailless version FcγRIIa (*lanes 5* and *6*) along with SHIP. Forty-eight hours after transfection cells were harvested and activated by clustering FcγRIIa with IV.3 Fab and GAM. SHIP was immunoprecipitated from resting and activated cells and analyzed for tyrosine phosphorylation by Western blotting with anti-phosphotyrosine Abs. As the results indicate (Fig. 1*A*, *upper panel*), SHIP phosphorylation was induced by FcγRIIa in a manner that is dependent on the expression of the ITAM present in the cytoplasmic tail of the receptor (*lane 4* but not *lane 6*). The membrane was reprobed with anti-SHIP Ab to ensure comparable levels of SHIP expression and to affirm that the phosphorylation signal observed was not due to a difference in amounts of protein loaded in the different lanes. In additional experiments, P388D1 mouse macrophage cells stably transfected with human FcγRIIa were activated by clustering

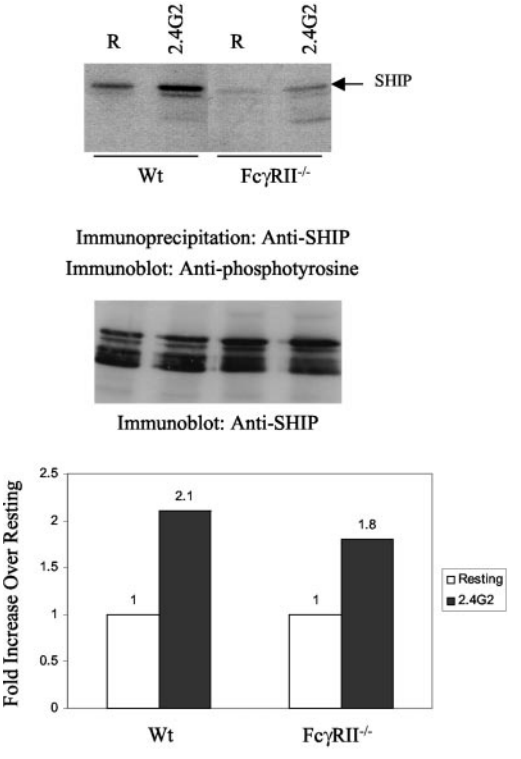

FIGURE 2. SHIP phosphorylation is induced by the ITAM-associated FcγRIIIa in FcγRII-deficient mice. BMMs derived from wild-type and FcγRII-deficient mice were activated by clustering FcγRIIIa/II with mAb 2.4G2 and mouse anti-rat IgG secondary Ab. SHIP proteins were immunoprecipitated from resting and activated cells and assayed for tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine Ab (*upper panel*). The membrane was reprobed with anti-SHIP Ab to ensure equal loading in all lanes (*middle panel*). Phosphorylation of SHIP is expressed as fold increase over that seen in resting cells (*lower panel*). These results are representative of two independent experiments.

FcγRIIa. SHIP phosphorylation was assessed by Western blotting. As seen in Fig. 1*B*, *upper panel*, SHIP is tyrosine phosphorylated in cells activated by clustering FcγRIIa (*lane 2*). Shown in Fig. 1*B*, *lower panel*, is a reprobe with anti-SHIP Ab to indicate equal loading of SHIP in all lanes.

Second, to assess whether FcγRI can induce SHIP phosphorylation in the absence of FcγRIIb, COS-7 cells were transiently transfected to express FcγRI α-chain and SHIP (Fig. 1*C*, *lanes 1* and *2*) or FcγRI α-chain, the γ subunit, and SHIP (*lanes 3* and *4*). Cells were activated by clustering FcγRI with $F(ab')_2$ of mAb 32.2 and GAM, followed by immunoprecipitation with anti-SHIP Ab. SHIP phosphorylation was assessed as described above. Results indicated that indeed FcγRI could induce SHIP phosphorylation when the ITAM-bearing γ subunit was coexpressed (*lane 3*), but not when the FcγRI α-chain was expressed alone (*lane 2*).

Third, we assessed the ability of murine ITAM-FcγR to induce SHIP phosphorylation in the absence of FcγRIIb. For this, BMMs derived from wild-type and FcγRII-deficient mice were activated by clustering FcγR with the mAb 2.4G2 that recognizes equally well the ITAM-bearing FcγRIIIa and the ITIM-bearing FcγRII. SHIP phosphorylation was assessed by Western blotting with anti-phosphotyrosine Ab (Fig. 2, *upper panel*). Results indicated that, while SHIP phosphorylation was maximally induced in cells expressing FcγRII (Fig. 2, *lane 2*), FcγRIIIa alone induced measurable tyrosine phosphorylation of SHIP (Fig. 2, *lane 4*). These data collectively provide compelling evidence that ITAM-FcγR are capable of inducing SHIP phosphorylation in vivo.

SHIP coimmunoprecipitates with endogenous FcγRIIa

We and others have earlier reported that SHIP is capable of associating in vitro with phosphorylated ITAM peptides derived from FcγRIIa (28), the γ subunit of FcεRI, FcγRI, and IIIa, and the ζ subunit of TCR (31). To test whether SHIP could associate in vivo with an ITAM, we performed coimmunoprecipitation experiments in THP-1 cells, testing here the ability of endogenous SHIP to interact with endogenous FcγRIIa. For this, FcγRIIa was immunoprecipitated from resting (Fig. 3*A*, *upper panel*, *lane 1*) and activated (*lane 2*) THP-1 cells that were activated by clustering FcγRIIa receptors. FcγRIIa immunoprecipitates were analyzed for the presence of SHIP in the resultant immune complex by Western blotting with anti-SHIP Ab. As seen in Fig. 3*A*, SHIP protein was detectable in FcγRIIa immunoprecipitates only upon activation of the cells (*lane 2*). The same membrane was reprobed for FcγRIIa (Fig. 3*A*, *lower panel*).

As an additional approach to determine whether SHIP-FcγRIIa interaction occurs in vivo, COS-7 cells were transfected with empty vectors (Fig. 3*B*, *upper panel*, *lanes 1* and *2*) or with plasmids encoding FcγRIIa and SHIP (*lanes 3* and *4*). Cells were activated by clustering FcγRIIa (*lanes 2* and *4*). FcγRIIa was immunoprecipitated and analyzed for association with SHIP by Western blotting with anti-phosphotyrosine Ab. As seen in Fig. 3*B*, *upper panel*, receptor clustering induced phosphorylation of FcγRIIa and association with p145 SHIP. The membrane was subsequently reprobed with anti-SHIP (*middle panel*) and anti-FcγRIIa (*lower panel*).

SHIP is capable of direct association with FcγRIIa ITAM in vitro

To assess whether the association of SHIP with FcγRIIa is direct or is mediated by an adapter protein we first performed a far Western analysis applying a phosphorylated FcγRIIa ITAM peptide (pITAM) to SHIP immunoprecipitates on nitrocellulose membrane. As the results indicate (Fig. 4*A*, *upper panel*), pITAM bound SHIP (*lane 1*), Shc, and the p85 subunit of phosphatidylinositol 3-kinase (*lanes 2* and *3*) but not Grb2 (*lane 4*). The membrane was reprobed with anti-Grb2 Abs to indicate that Grb2 was indeed present on the membrane (*lower panel*, *lanes 1* and *4*) but the ITAM peptide was incapable of binding to it. This result is consistent with previous reports indicating that immunoreceptors, unlike growth factor receptors, do not bind Grb2 directly; rather, they do so through Shc (32). The anti-Grb2 reactive band in *lane 1* indicates that Grb2 is present in the SHIP immunoprecipitate as previously reported (reviewed in Ref. 33). A parallel membrane probed with a phosphorylated ITIM peptide (pITIM) derived from FcγRIIb indicated that the ITIM was capable of strong binding to SHIP but not with Shc, p85, or Grb2 (Fig. 4*B*). However, it is noteworthy that the direct interaction of pITAM with SHIP was severalfold weaker than that of pITIM. In contrast, the interaction of pITAM with Shc and p85 was much stronger than the interaction of pITAM-SHIP. These results prompted us to investigate the

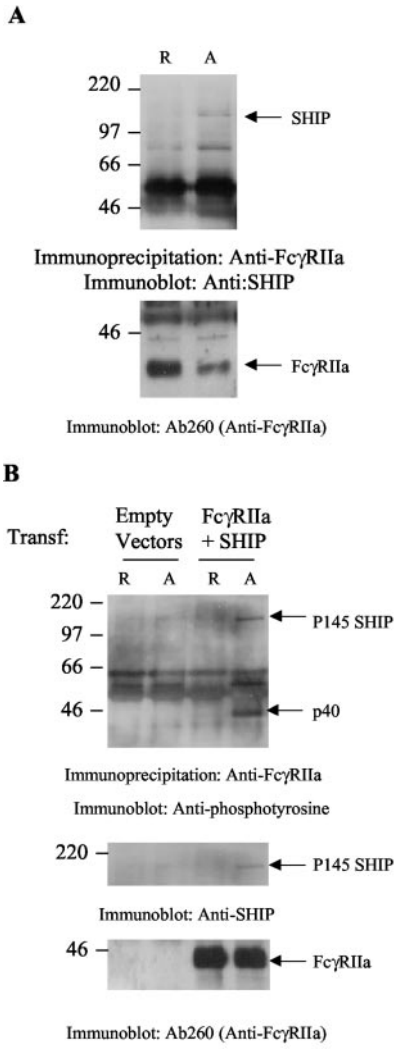

FIGURE 3. SHIP associates with FcγRIIa in vivo. *A*, FcγRIIa was immunoprecipitated from resting and activated THP-1 cells that were activated by clustering FcγRIIa (*lanes 1* and *2*). The membrane was probed with anti-SHIP Ab (*upper panel*) and subsequently with anti-FcγRIIa Ab (*lower panel*). *B*, COS-7 cells were either transfected with vector alone or transfected with plasmids encoding FcγRIIa and SHIP. Cells were activated by clustering FcγRIIa followed by immunoprecipitation of FcγRIIa. The membrane was probed with anti-phosphotyrosine (*upper panel*). The same membrane was subsequently reprobed with anti-SHIP (*middle panel*), and with Ab260 to detect the presence of FcγRIIa in the immunoprecipitates (*lower panel*).

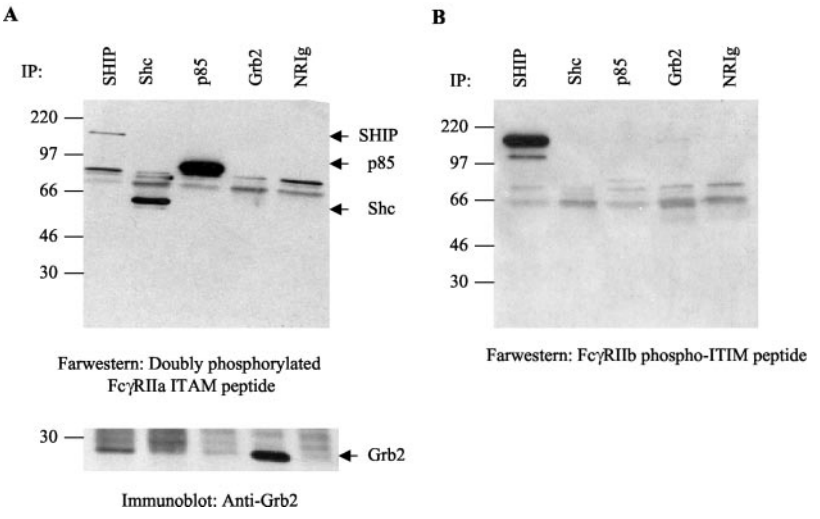

FIGURE 4. SHIP is capable of direct association with FcγRIIa ITAM peptide. SHIP, Shc, p85, Grb2, and normal rabbit Ig immunoprecipitates from THP-1 cells were separated on SDS-PAGE, transferred to nitrocellulose membranes, and probed with either a phosphorylated FcγRIIa ITAM peptide (*A, upper panel*) or a phosphorylated FcγRIIb ITIM peptide (*B*). The membrane in *A* was reprobed with anti-Grb2 Ab (*A, lower panel*).

possibility that the in vivo association of SHIP with FcγRIIa might be facilitated via adapter molecules rather than by a direct interaction between SHIP and the receptor.

Dominant negative Shc but not Grb2 attenuates SHIP tyrosine phosphorylation by FcγRIIa

SHIP is reported to associate with at least two adapter molecules: Shc and Grb2 (reviewed in Ref. 33). The interaction of SHIP with Grb2 is constitutive and occurs via the Grb2 SH3 domain and the C-terminal proline-rich domain of SHIP. In contrast, the association of SHIP with Shc is activation induced, requiring tyrosine phosphorylation events and involving multiple interaction domains (34). Studies in B cells indicated that the SHIP SH2 domain can interact with the phosphotyrosines of Shc and in turn the Shc phosphotyrosine binding domain can interact with the phosphotyrosines of SHIP (14). To test whether the in vivo interaction of SHIP with FcγRIIa is direct or whether it occurs via adapter proteins such as Shc and Grb2, we used a transient transfection system. Here we transfected into COS-7 cells plasmids encoding FcγRIIa alone (Fig. 5*A*, *lanes 1* and *2*), FcγRIIa and SHIP (*lanes 3* and *4*), FcγRIIa, SHIP and a dominant negative Grb2 that is mutated in its 2 SH3 domains (*lanes 5* and *6*), or with FcγRIIa, SHIP, and a dominant negative mutant of Shc (*lanes 7* and *8*). The Shc mutant has all three phosphorylatable tyrosine residues (Y239, Y240, and Y317) replaced by phenylalanine. Empty vectors were used to equalize amounts of DNA used for transfection. Transfected cells were harvested 48 h later and SHIP proteins were immunoprecipitated from resting cells and cells activated by clustering FcγRIIa. Western blotting with anti-phosphotyrosine Ab (Fig. 5*A*, *upper panel*) indicated that SHIP phosphorylation is attenuated in cells overexpressing a dominant negative Shc (*lane 8*) but not in cells expressing dominant negative Grb2 (*lane 6*). These results are representative of four independent experiments. The membrane was reprobed with anti-SHIP Ab to ensure equal loading of SHIP in all lanes (*lower panel*). To demonstrate that the dominant negative Grb2 was indeed functional we analyzed Erk

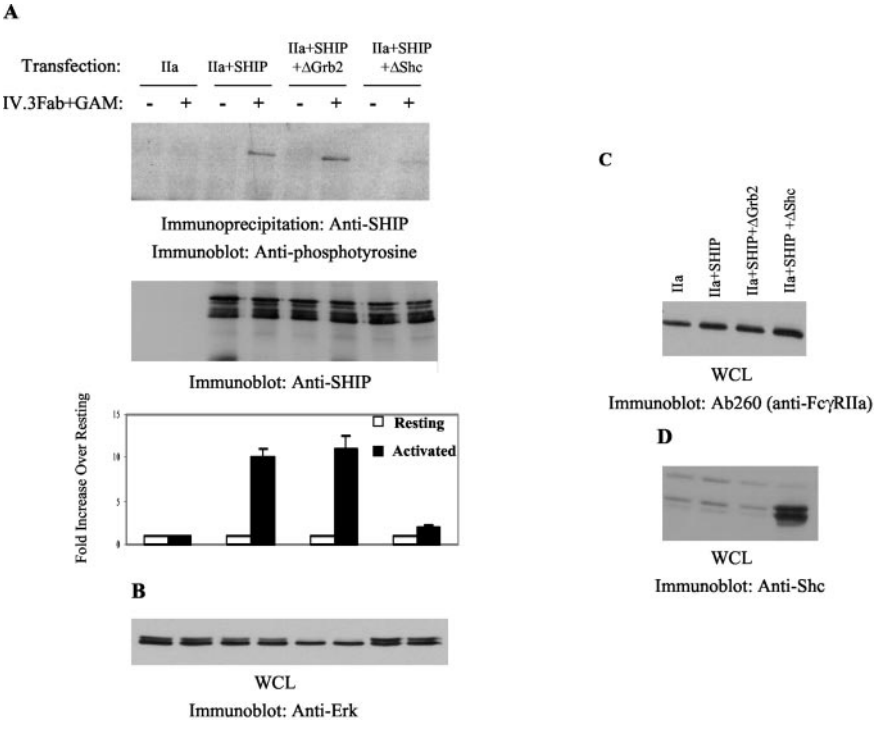

FIGURE 5. Overexpression of dominant negative Shc but not Grb2 attenuates SHIP phosphorylation by FcγRIIa. COS-7 cells were transfected to express the proteins indicated. Forty-eight hours after transfection, cells were activated by clustering FcγRIIa. *A*, SHIP proteins were immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine Ab (*upper panel*). The membrane was reprobed with anti-SHIP Ab (*middle panel*). SHIP phosphotyrosine signals were quantitated and expressed as fold increase of the activated cells over the phosphorylation in resting cells (*lower panel*). The graph represents the mean and SD from the means of two independent experiments. *B*, Whole cell lysates (WCL) from the same samples as above were probed with anti-Erk Ab to demonstrate the dominant negative effect of the transfected Grb2 mutant on the Ras signaling pathway in *lanes 5* and *6*. *C*, Whole cell lysates (WCL) from the transfectants were probed with anti-FcγRIIa Ab to ensure expression of the transfected receptor. *D*, Whole cell lysates (WCL) were probed with anti-Shc Ab to demonstrate overexpression of Shc.

activation in the transfected COS-7 cells. As seen in Fig. 5*B*, Erk is heavily activated in these cells, as indicated by the gel shift, in all cases except when the mutant Grb2 is overexpressed. The activation of Erk seen here is constitutive (likely induced by growth factors in the serum) and is independent of FcγRIIa clustering. Fig. 5*C* shows equal expression of FcγRIIa in all transfectants and Fig. 5*D* demonstrates overexpression of transfected Shc. We conclude, based on the above data, that SHIP phosphorylation by FcγRIIa requires Shc, suggesting that the association of SHIP with FcγRIIa is via Shc.

SHIP SH2 domain is necessary for SHIP phosphorylation by FcγRIIa

The above data suggest a model in which Shc associates with the phosphorylated ITAM of FcγRIIa, becomes phosphorylated on tyrosine residues, and serves as a docking site for SHIP SH2. The SHIP proteins that are thus transported to the membrane are then phosphorylated by the membrane-associated Src kinases, as previously reported (35). To test whether SHIP SH2 domain is involved in the translocation of SHIP to the receptor and the subsequent tyrosine phosphorylation of SHIP, COS-7 cells were transiently transfected to express SHIP alone (Fig. 6*A*, *lanes 3* and *4*), FcγRIIa and SHIP (*lanes 5* and *6*), or FcγRIIa, SHIP, and excess of SHIP SH2 domain (*lanes 7* and *8*). SHIP phosphorylation in response to FcγRIIa clustering was assessed in the transfectants by Western blotting. Consistent with our model, overexpression of SHIP SH2 domain attenuated FcγRIIa-induced SHIP phosphorylation (Fig. 6*A*, *lane 8*). The membrane was reprobed with anti-SHIP Ab to ensure that the lack of phosphorylation in *lane 8* was not due to differences in the amount of SHIP protein present in the resting and activated lanes (Fig. 6*A*, *lower panel*). To exclude the possibility that the lack of SHIP phosphorylation in the SHIP SH2 overexpressing cells is not due to a lack of FcγRIIa expression, whole cell lysates from the transfectants were analyzed by Western blotting for the presence of transfected FcγRIIa (Fig. 6*B*). Results indicate that FcγRIIa is indeed expressed in all cells transfected with a plasmid encoding FcγRIIa (*lanes 3* and *4*, but not *lanes 1* and *2*); therefore, the lack of SHIP phosphorylation upon receptor clustering could be due only to the dominant negative effect of the overexpressed SHIP SH2 domain.

SHIP regulates NF-κB induction by ITAM-FcγR

The functional consequence of SHIP activation by phagocytic FcγR is not clear. Because inflammatory cytokine genes, such as IL-1, IL-8, and TNF-α, that are induced during phagocytosis require the activation of NF-κB (36), we asked whether SHIP activation would regulate NF-κB-dependent gene transcription by phagocytic FcRs. In these experiments we analyzed NF-κB-dependent transcription from a reporter plasmid encoding the luciferase gene in the presence of excess of wild-type SHIP or the dominant negative SHIP SH2 domain that abrogates association of SHIP with the ITAM, as we have demonstrated above. Thus, THP-1 cells were transiently cotransfected with NF-κB-luc plasmid and with plasmids encoding wild-type SHIP or SHIP SH2 domain. The transfected cells were activated by clustering either FcγRI (Fig. 7*A*) or FcγRIIa (Fig. 7*B*) for 6 h at 37°C by methods described above. Transcription of the luciferase gene was measured by a luciferase enzyme assay, as previously described (36). Results are expressed as fold increase in luciferase activity in cells activated by clustering FcRs over the activity in resting cells. Data indicate that NF-κB-dependent transcription of the luciferase gene

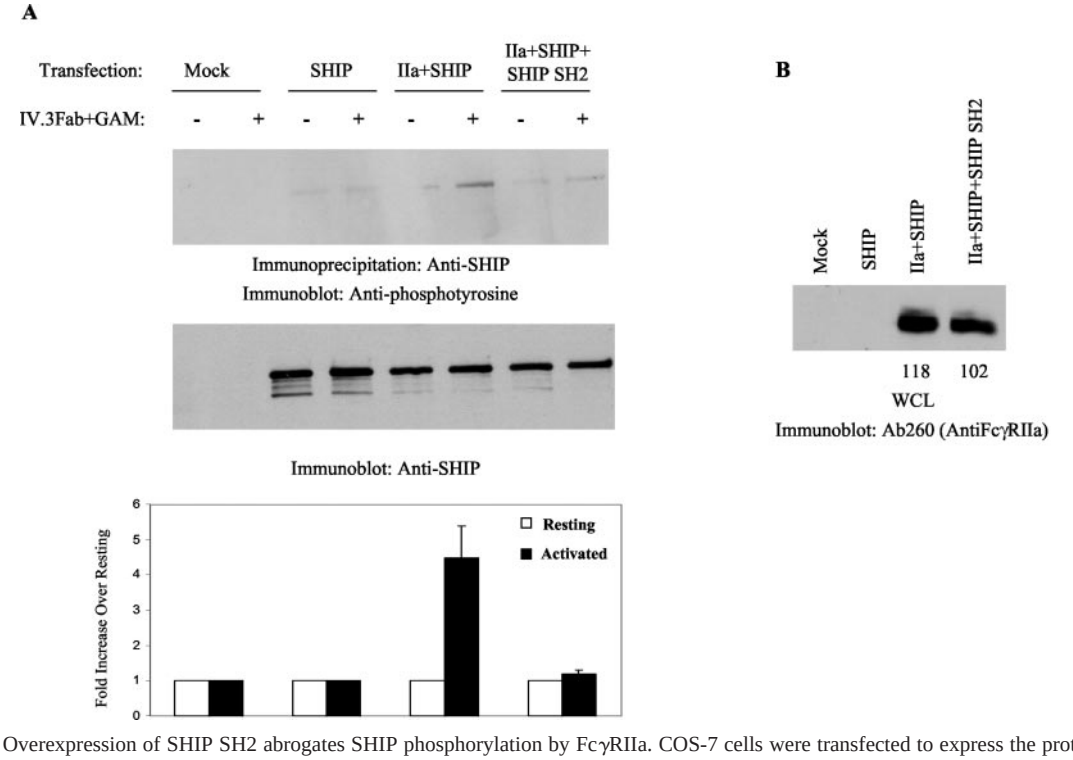

FIGURE 6. Overexpression of SHIP SH2 abrogates SHIP phosphorylation by FcγRIIa. COS-7 cells were transfected to express the proteins indicated. Cells were harvested 48 h posttransfection and activated by clustering FcγRIIa. *A*, SHIP proteins were immunoprecipitated and analyzed by Western blotting with anti-phosphotyrosine Ab (*upper panel*). The membrane was reprobed with anti-SHIP Ab (*middle panel*). SHIP phosphotyrosine signals were quantitated and expressed as fold increase of the activated cells over the phosphorylation in resting cells (*lower panel*). The graph represents the mean and SD from the means of two independent experiments. *B*, Whole cell lysates (WCL) from the transfectants were probed with anti-FcγRIIa Ab to ensure expression of the transfected receptor. The numbers *below* the panel indicate densitometry measurement of band intensity.

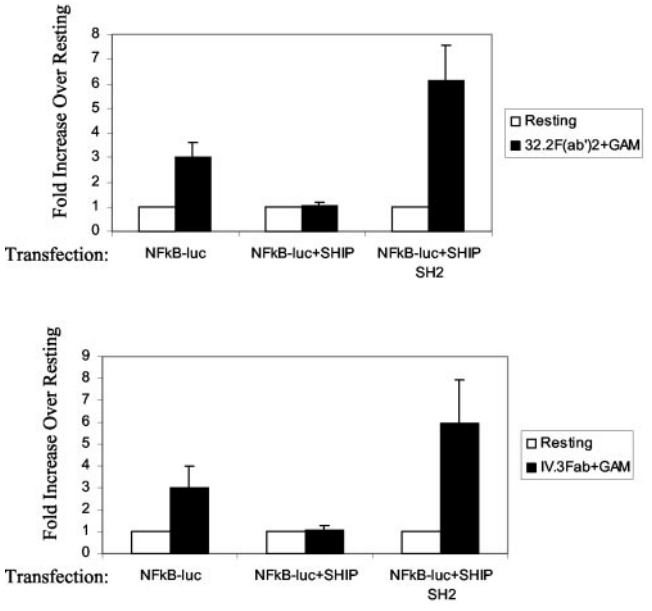

FIGURE 7. SHIP down-regulates NF-κB-dependent gene transcription mediated by phagocytic FcγR. THP-1 cells were transfected with the plasmids indicated. Transfectants were activated by clustering either FcγRI or FcγRIIa with mAb 32.2 $F(ab')_2$ plus GAM or IV.3 Fab plus GAM, respectively, for 6 h. Cells were then lysed and analyzed for luciferase activity. Data are expressed as fold increase of luciferase activity in the activated cells over that in resting cells. The graphs represent the mean and SD of three independent experiments.

occurs upon FcR clustering (~3-fold induction). However, overexpression of wild-type SHIP blocks the induction of gene transcription. Importantly, gene transcription is greatly enhanced in the presence of a dominant negative SHIP SH2 domain (~6-fold induction), suggesting that SHIP serves to down-regulate NF-κB-dependent gene transcription by phagocytic FcγR.

Discussion

IgG-coated infectious particles engage both activating and inhibitory FcγR expressed on the cell surface of macrophages/monocytes, leading to the activation of multiple signaling pathways that result in the ingesting, transport to the lysosome, and subsequent degradation of the infectious particle by lysosomal enzymes. Because the process of phagocytosis results in the production of tissue-damaging products such as reactive oxygen and nitrogen species, inflammatory cytokines, and lysosomal enzymes, a tight control on the magnitude of the phagocytic response is essential. Regulation of FcγR-mediated phagocytosis is achieved at several levels. First, the ratio of activating and inhibiting FcγR on the cell surface determines the magnitude of the phagocytic response. Thus, macrophages from mice deficient in FcγRIIb expression display enhanced phagocytic capability in comparison to wild-type macrophages (25). Likewise, monocytes cultured in the presence of IL-4, which express more FcγRIIb, are less efficient at phagocytosing IgG-coated particles than monocytes cultured in the absence of IL-4 (5, 6). Second, the expression and activation of SHIP influences phagocytic efficiency. Macrophages derived from SHIP-deficient mice are better able to phagocytose IgG-coated particles than are their wild-type counterparts (7). Because SHIP activation has been demonstrated to be mediated not only by the ITIM-bearing FcγRIIb but also by ITAM-bearing receptors, it is a fascinating possibility that the levels of SHIP at the cell membrane are highly variable, thus providing a finely tuned regulatory mechanism for cellular responses such as phagocytosis.

The molecular mechanisms leading to the association of SHIP with the phosphorylated ITIM of FcγRIIb and the subsequent phosphorylation of SHIP are well characterized (37, 38). SHIP is a constitutively active cytosolic enzyme that requires membrane translocation to gain access to its lipid substrates (35). Recruitment of SHIP to the membrane is also obligatory for tyrosine phosphorylation of SHIP. Thus, tyrosine phosphorylation of SHIP serves as a good indicator of membrane translocation of the enzyme. We and others have previously reported that SHIP phosphorylation is achieved when ITAM-FcγR are clustered on monocytes. However, the molecular details of SHIP phosphorylation by activating receptors are poorly understood.

The results of this study indicate that SHIP phosphorylation by the ITAM-associated FcγRIIa requires Shc phosphorylation, leading to the model presented in Fig. 8. Thus, clustering of FcγRIIa leads to the activation of Src kinases that phosphorylate the tyrosines in the ITAM of the receptor. The phosphorylated ITAM serves as a binding site for the SH2 domain of Shc, which is itself subsequently phosphorylated on tyrosine residues. The direct association of Shc with ITAMs has previously been reported (32). The phosphotyrosine residues of Shc engage the SHIP SH2 domain, thus bringing SHIP to the membrane, where SHIP is phosphorylated by the membrane-associated Src kinases (35). Our previous findings that a phosphorylated ITAM peptide from FcγRIIa is able to associate with SHIP in resting cell lysates where Shc is not tyrosine phosphorylated (28), as well as the Far Western analysis presented in Fig. 4, suggest a possible direct interaction of SHIP with FcγRIIa ITAM. However, when this latter model was tested in an in vivo assay, presented in Fig. 5, it became very apparent that a direct interaction between SHIP and the FcγRIIa ITAM is unlikely to occur in vivo. The latter may be due to the presence of other SH2 domain-containing molecules in the cell

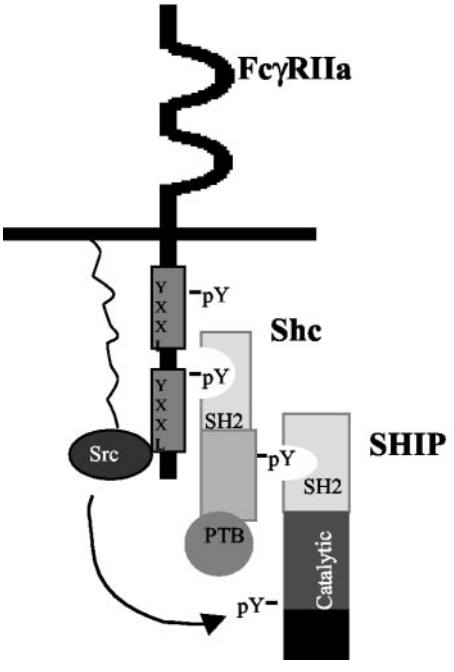

FIGURE 8. Model for SHIP association with the FcγRIIa ITAM. FcγRIIa clustering leads to the activation of Src kinases that phosphorylate the ITAM in the receptor. Shc associates with the phosphorylated ITAM via the Shc SH2 domain and is subsequently phosphorylated on tyrosines 239, 240, and 317. The phosphotyrosines of Shc serve to dock the SHIP SH2 domain. The SHIP molecule that is thus localized in the membrane is phosphorylated by the Src kinases.

lysate that have a higher affinity for the ITAM than the SHIP SH2. Were it possible for SHIP to interact directly with FcγRIIa, the overexpression of the Shc mutant would have had no influence on FcγRIIa-induced SHIP phosphorylation; i.e., phosphorylation of SHIP in Fig. 5*A*, *upper panel*, *lane 8*, would have paralleled the phosphorylation of SHIP seen in *lanes 4* and *6*. A third potential model would present the following scenario: Shc associates with phosphorylated ITAM of FcγRIIa, Shc becomes phosphorylated, and Grb2 SH2 domain associates with phospho-Shc and brings along SHIP, which is attached to the Grb2 SH3 domain. This last model is again unlikely to account for SHIP phosphorylation by FcγRIIa, because a dominant negative double SH3 Grb2 mutant failed to influence SHIP phosphorylation (Fig. 5*A*, *lane 6*).

Although the model presented in Fig. 8 accounts for our current observations, there are several intriguing questions about the signaling pathways that arise from this work that are likely to reveal novel mechanisms used for regulation of cell activation. For example, studies from our group and others previously reported that the SHIP SH2 domain and the Grb2 SH2 domain compete for the same phosphotyrosine residues on Shc (34, 39); however, surface plasmon resonance analysis revealed that the affinity of the Grb2 SH2 domain for phospho-Shc is higher than the affinity of SHIP SH2 domain for phospho-Shc (23). The higher affinity of Grb2 SH2 for phospho-Shc explains our earlier findings that SHIP phosphorylation achieved by clustering ITAM-FcγR is severalfold lower than the SHIP phosphorylation that occurs when ITAM-FcγR are coclustered with FcγRIIB. However, it is not clear why the Grb2 molecules do not serve to adapt SHIP to Shc. Grb2 is reported to associate with a number of proline-rich domain-containing proteins such as the Ras guanine exchange factor Sos, SHIP, and Cbl, via the Grb2 SH3 domain. It is possible that the affinity of Grb2 SH3 binding with SHIP is not strong enough to compete with the other Grb2-binding partners. Alternately, it is possible that the numbers of molecules of SHIP are far fewer than the other Grb2-binding proteins. Further analysis is required to resolve these issues.

Based on the model shown in Fig. 8, we have interrupted SHIP function with a dominant negative SHIP SH2 domain in transiently transfected THP-1 cells and analyzed the outcome of SHIP function on FcγR-mediated outcome. Our results indicate that SHIP regulates NF-κB-mediated gene transcription. These results are consistent with a recent report by Kalesnikoff et al. (40), which demonstrated a regulatory role for SHIP in IgE-mediated IL-6 production by inhibiting NF-κB activation. SHIP hydrolysis of phosphatidylinositol 3,4,5 trisphosphate leads to the down-regulation of a number of signaling pathways, including the activation of Akt, Erk, and p38, all of which are implicated in the activation of NF-κB (40).

In conclusion, in a study aimed at understanding regulation of FcγR-mediated activation, we demonstrate here that ITAM-FcγR are capable of inducing SHIP phosphorylation in myeloid cells. We have dissected the molecular events that lead to SHIP tyrosine phosphorylation by FcγRIIa and report that the adapter molecule Shc, but not Grb2, is necessary for phosphorylation of SHIP by FcγRIIa. These studies are the first to demonstrate that SHIP associates with and is recruited by native ITAM-bearing receptors independently of FcγRIIb involvement. The functional consequence of SHIP activation by ITAM-FcγR is not known. Using the information gained from the above experiments we have analyzed the influence of SHIP on gene transcription induced by phagocytic FcγR under conditions that preclude the activation of SHIP by these receptors. Our data suggest that SHIP serves to down-regulate NF-κB-dependent gene transcription during the phagocytic process, thus providing a mechanism to limit the inflammatory processes associated with phagocytosis.

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