The RasGAP-Binding Protein p62dok Is a Mediator of Inhibitory FcγRIIB Signals in B Cells

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Summary

The low affinity receptor for IgG, FcγRIIB, functions to dampen the antibody response and reduce the risk of autoimmunity. This function is reportedly mediated in part by inhibition of B cell antigen receptor (BCR)-mediated $p21^{ras}$ activation, though the basis of this inhibition is unknown. We show here that FcγRIIB-BCR coaggregation leads to increased tyrosine phosphorylation of the RasGAP-binding protein p62dok, with a concomitant increase in its binding to RasGAP. These effects require the recruitment and tyrosine phosphorylation of the phosphatidylinositol 5-phosphatase SHIP, which further recruits p62dok via the latter's phosphotyrosine-binding domain. Using chimeric FcγRIIB containing the RasGAP-binding domain of p62dok, we demonstrate that p62dok contains all structural information required to mediate the inhibitory effect of FcγRIIB on Erk activation.

Introduction

Signaling through a variety of antigen receptors, including B cell receptor (BCR), T cell receptor (TCR), FcεRI (Daeron et al., 1995), and FcγRIII (Takai et al., 1996), is inhibited by coaggregation of FcγRIIB, a receptor for immunoglobulin constant region. Physiologically, coaggregation of FcγRIIB with BCR occurs by the simultaneous binding of immune complexes to the antigen receptor and FcγRIIB (through antigenic epitopes and IgG Fc determinants, respectively) (Morgan and Tempelis, 1978). Recent studies demonstrate that this mechanism serves to reduce the likelihood of developing autoimmunity and the severity of anaphylactic responses (Takai et al., 1996; Yuasa et al., 1999).
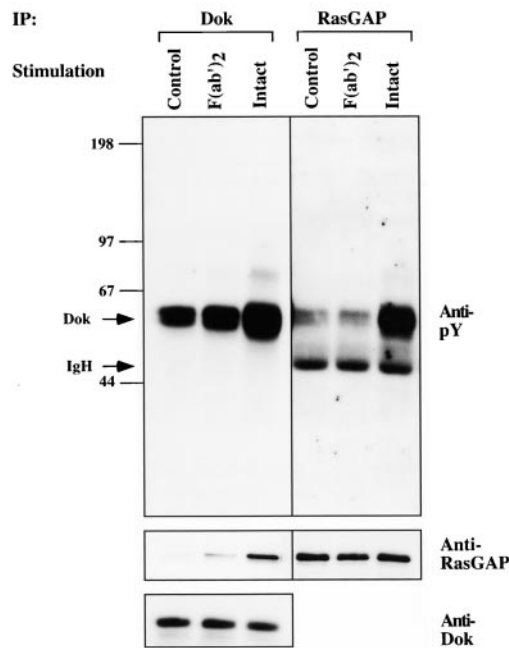
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The molecular circuitry by which FcγRIIB inhibits BCR signaling is still unclear. FcγRIIB signaling requires a cytoplasmic domain motif (Amigorena et al., 1992; Muta et al., 1994; Daeron et al., 1995) that is conserved among inhibitory receptors. This domain, the immunoreceptor tyrosine-based inhibitor motif (ITIM), undergoes tyrosine phosphorylation upon FcγRIIB coaggregation with BCR in B cells (Muta et al., 1994). This phosphorylation creates a binding site for the SH2 domains of three phosphatases, SHP-1 and SHP-2 (KIR and FcγRIIB) and the phosphatidylinositol phosphate 5-phosphatase SHIP (FcγRIIB) (D'Ambrosio et al., 1995). Binding of these effectors to the phosphorylated ITIM motif brings them in close proximity to the coligated activating receptor, allowing exercise of the inhibitory activity of the phosphatases. SHIP, which is the primary effector of FcγRIIB (Fong et al., 1996; Ono et al., 1996, 1997; Gupta et al., 1997), dephosphorylates phosphoinositides and inositol polyphosphates at their 5 position (Kavanaugh et al., 1996). Its major in vivo substrate is phosphatidylinositol-3,4,5-triphosphate (PIP_3), formed by the action of phosphatidylinositol 3-kinase (PI3-K) on phosphatidylinositol-4,5-biphosphate (PIP_2) (Damen et al., 1996; Lioubin et al., 1996). The importance of PIP_3 in BCR signal transduction is underscored by its role in translocation of key signal transducing components, including phospholipase C gamma (PLCγ) (Falasca et al., 1998) and Bruton's tyrosine kinase (Btk) (Salim et al., 1996; Bolland et al., 1998; Scharenberg et al., 1998), to the plasma membrane. Hence, SHIP is believed to modulate signal transduction by hydrolyzing PIP_3, preventing the proper assembly of a signaling complex capable of mediating PIP_2 hydrolysis and Ca^{2+} mobilization (Bijsterbosch and Klaus, 1985; Wilson et al., 1987; Ono et al., 1996).

FcγRIIB coaggregation also inhibits BCR activation of Ras (Sarmay et al., 1996). The basis of this effect is still unclear. Upon FcγRIIB-BCR coaggregation, SHIP is tyrosine phosphorylated and associates with Shc, a Ras pathway adaptor (Tridandapani et al., 1997a). It has been suggested that SHIP competes with Grb2-Sos complexes for Shc binding and this is responsible for the observed decrease in Ras-bound GTP detectable upon FcγRIIB-BCR coaggregation (Tridandapani et al., 1997b). This model is also consistent with the observation that Erk phosphorylation is inhibited upon FcγRIIB-BCR coaggregation in wild-type (wt) but not SHIP-deficient B cells (Liu et al., 1998).
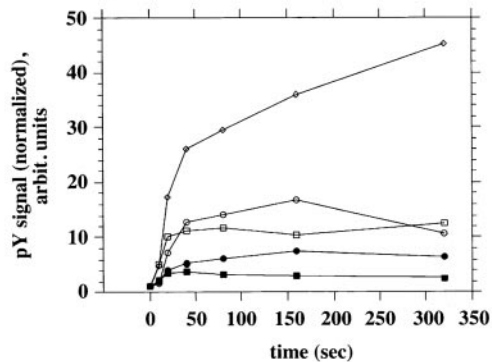
The GTPase-activating protein RasGAP has also been implicated in Ras regulation (Gold et al., 1993; Lazarus et al., 1993). This protein acts by enhancing the intrinsic GTPase activity of Ras, leading to hydrolysis of bound GTP to GDP. The recently cloned adaptor protein p62dok (Carpino et al., 1997; Yamanashi and Baltimore, 1997) associates with RasGAP upon BCR aggregation (Gold et al., 1993). This association is believed to involve phosphotyrosyl residues in p62dok that bind SH2 domain(s) in RasGAP (Yamanashi and Baltimore, 1997). The increased tyrosine phosphorylation of p62dok has been observed in cells expressing the viral oncogene protein

A.

IP: Dok · RasGAP

Stimulation: Control · F(ab')2 · Intact · Control · F(ab')2 · Intact

198 —
97 —
67 —
Dok →
IgH →
44 —

Anti-pY

Anti-RasGAP

Anti-Dok

B.

pY signal (normalized), arbit. units

50
40
30
20
10
0

0 50 100 150 200 250 300 350

time (sec)

Figure 1. Increased p62^{dok} Tyrosine Phosphorylation and Association with RasGAP upon FcγRIIB1 Coaggregation with BCR

(A) FcγRIIB1-BCR coaggregation in splenic B cells leads to increased tyrosine phosphorylation of p62^{dok} and its association with RasGAP. Splenic B cells were stimulated with IgM-specific rabbit antibody (Intact) or its F(ab')$_2$ for 4 min at 37°C and lysed. Lysates were immunoprecipitated (IP) with RasGAP- and p62^{dok}- (Dok) specific antibodies followed by SDS-PAGE, electrophoretic transfer to PVDF membranes, and immunoblot analysis using phosphotyrosine- (pY), Dok- or RasGAP-specific antibodies (as indicated). Arrows mark positions of major species.

(B) Time course of SHIP, p62^{dok}, and FcγRIIB1 tyrosine phosphorylation. A20 B lymphoma cells were stimulated by an IgG-specific rabbit antibody or its F(ab')$_2$ for the indicated duration, followed by lysis and immunoprecipitation with SHIP-, p62^{dok}-, or FcγRIIB1-specific antibodies. Precipitates were resolved by SDS-PAGE followed by electrophoretic transfer and immunoblotting using phosphotyrosine- (pY), SHIP-, Dok-, or FcγRIIB1-specific antibodies (as indicated). Blots were scanned and the results analyzed using a densitometry program (ScanAnalysis, Biosoft). The phosphotyrosine

tyrosine kinases v-Abl, v-Src, v-Fps, and v-Fms, suggesting it plays a role in their transformation (Ellis et al., 1990). Moreover, the transforming chimeric kinase Bcr-Abl has been shown to bind and strongly induce the tyrosine phosphorylation of p62^{dok}, suggesting a possible global and positive role for p62^{dok} in mitogenic signaling (Carpino et al., 1997).

We show here that the tyrosine phosphorylation of p62^{dok} and its binding to RasGAP are strongly potentiated by FcγRIIB-BCR coaggregation. This response is correlated with SHIP phosphorylation and formation of a p62^{dok}-SHIP complex, mediated by interaction between phosphotyrosyl residues of SHIP and the phosphotyrosine-binding (PTB) domain in p62^{dok}. SHIP expression was indispensable for FcγRIIB-BCR coaggregation-induced tyrosine phosphorylation of p62^{dok}, indicating that SHIP serves as the primary adaptor for FcγRIIB-mediated activation of p62^{dok}. Perhaps most importantly, when coaggregated with BCR, a chimeric receptor containing the C-terminal RasGAP-binding region of p62^{dok} inhibited Erk activation. This implicates p62^{dok} as a key intermediary in FcγRIIB inhibition of BCR-mediated activation of the Ras signaling pathway.

Results

FcγRIIB1 Coaggregation with BCR Leads to Increased p62^{dok} Tyrosine Phosphorylation and Association with RasGAP

In the course of our studies of the effects of FcγRIIB1 coaggregation with BCR on the tyrosine phosphorylation of target proteins, we observed an FcγRIIB1-dependent increase in the phosphorylation of an undefined 60–65 kDa protein and hence explored the possibility that this protein is p62^{dok}. Both RasGAP and p62^{dok} immunoprecipitates of primary splenic B cells contained a 62 kDa protein whose tyrosine phosphorylation was increased upon cell treatment with an IgM-specific polyclonal antibody (intact RAMμ) that coaggregated BCR with FcγRIIB1 (Figure 1A). Cell pretreatment with an FcγRIIB1-blocking rat mAb (2.4G2) prior to RAMμ or stimulation with F(ab')$_2$ fragments of RAMμ resulted in a much less robust p62^{dok} tyrosine phosphorylation (data not shown). More striking was the induction of RasGAP association with p62^{dok} upon BCR-FcγRIIB1 coaggregation (Figure 1A). Although significant basal and BCR-mediated tyrosine phosphorylation of p62^{dok} were observed in many experiments, p62^{dok} association with RasGAP was strongly dependent on BCR-FcγRIIB1 coaggregation. Depletion of RAMμ-stimulated cell lysates with the p62^{dok}-specific antibody resulted in a significant decrease (>80%) in the intensity of the 60–65 kDa phosphotyrosine signal, confirming its identity (data not shown).

signal at each time point and stimulation condition was field background-subtracted and normalized to the amount of precipitated protein, and ratio at t = 0 was set to 1 for each of the precipitated proteins. Shown are fold changes in normalized signal at t > 0. Unfilled shapes, stimulation with intact antibody; filled shapes, stimulation with F(ab')$_2$. Precipitation performed using p62^{dok}- (squares), SHIP- (circles), or FcγRIIB1-specific (diamonds) antibodies.

SHIP, p62[dok], and FcγRIIB1 are Tyrosine Phosphorylated with Similar Kinetics following FcγRIIB1 Coaggregation with BCR

To begin to establish the linkage between BCR-FcγRIIB1 coaggregation and p62[dok] function we studied the correlation among tyrosine phosphorylations of p62[dok], SHIP, and FcγRIIB1 in the FcγRIIB1-expressing lymphoma A20. The phosphotyrosine content of p62[dok] increased modestly (up to 4-fold) following BCR aggregation alone. However, a much larger increase (>10-fold) was observed following FcγRIIB1 coaggregation, and this increase was maximal at 40–80 s (Figure 1B). The rate of phosphorylation of both FcγRIIB1 and SHIP following BCR-FcγRIIB1 coaggregation was very similar to that of p62[dok]. The phosphotyrosine content of both p62[dok] and FcγRIIB1 remained at maximal levels even after 320 s of BCR-FcγRIIB1 coaggregation, while that of SHIP declined somewhat at the 320 s time point (Figure 1B). In contrast to FcγRIIB1, there was significant induction of tyrosine phosphorylation for both SHIP and p62[dok] following aggregation of BCR alone, with maximal phosphorylation reached between 1 and 2 min of stimulation in both cases.

The similarity in tyrosine phosphorylation kinetics of p62[dok], SHIP, and FcγRIIB1 suggested that all function very early following receptor coaggregation. The increase in phosphorylation of p62[dok] and SHIP but not FcγRIIB1 (see below and Figures 2A and 2B versus 2D) following BCR aggregation alone suggests that the tyrosine phosphorylation of p62[dok] and SHIP can also occur, albeit much less efficiently, via a BCR-autonomous mechanism.

The FcγRIIB1 ITIM Tyrosine Is Required for the Signaling-Dependent Increase in p62[dok] Tyrosine Phosphorylation and for p62[dok] Association with RasGAP

Previous studies (Chacko et al., 1996) have shown a requirement for FcγRIIB1 coaggregation for the efficient tyrosine phosphorylation of SHIP and demonstrated that SHIP association with FcγRIIB1 is mediated by the ITIM (D'Ambrosio et al., 1996). To explore the mechanistic linkage among the ITIM, SHIP, and p62[dok], we determined whether ITIM function is required for FcγRIIB1-mediated increases in p62[dok] phosphotyrosine content. Specifically, we examined whether the FcγRIIB1 ITIM tyrosine is required for phosphorylation of p62[dok]. We employed a B cell line (IIA1.6) transfected with FcγRIIB1 in which the ITIM tyrosine (Y309) was mutated to glycine (P. B. et al., unpublished data). This mutation abrogated FcγRIIB1-mediated increases in the phosphorylation of p62[dok] and SHIP (Figures 2A and 2B). However, phosphorylation of FcγRIIB1 Y309G was still observed, though much reduced (Figure 2D). Interestingly, the ITIM tyrosyl residue requirement for p62[dok]-RasGAP association appeared more stringent than that for p62[dok] tyrosine phosphorylation (Figure 2A versus 2C). Thus, enhanced tyrosine phosphorylation of SHIP and p62[dok] as well as p62[dok] association with RasGAP upon BCR-FcγRIIB1 coaggregation require the presence and thus probably the phosphorylation of the tyrosyl residue within the ITIM of FcγRIIB1.

FcγRIIB1-Mediated Tyrosine Phosphorylation of p62[dok] Requires SHIP Expression

Next we studied the mechanism by which FcγRIIB1 ITIM tyrosine phosphorylation leads to p62[dok] phosphorylation and association with RasGAP. Since p62[dok] lacks a domain that is predicted to associate directly with the phosphorylated ITIM, our data suggested a role for an ITIM binding protein, e.g., SHIP, as an adaptor for this association. Hence, we analyzed the effect of FcγRIIB1-BCR coaggregation on the induced tyrosine phosphorylation of p62[dok] in purified splenic B cells from SHIP^{−/−} mice. Despite comparable BCR, p62[dok] and FcγRIIB1 expression levels in splenic B cells obtained from wild-type and SHIP^{−/−} mice (Helgason et al., 1998; data not shown and Figure 3B, respectively), the FcγRIIB1-mediated tyrosine phosphorylation of p62[dok] was not seen in the latter (Figure 3A). In contrast, the tyrosine phosphorylation of FcγRIIB1 was comparably induced in B cells from SHIP^{−/−} and wild-type mice (Figure 3B). Taken together, these data implicate SHIP as the adaptor for p62[dok] in FcγRIIB1 signaling.

The Phosphoinositide Phosphatase SHIP Associates with p62[dok], Shc, and FcγRIIB1 upon FcγRIIB1-BCR Coaggregation

The relative molecular mass of the SHIP-associated phosphoproteins was consistent with those of Shc (53 kDa) and p62[dok] (62 kDa) (Figure 2B). To directly test their identity, we analyzed proteins that coimmunoprecipitate with SHIP, Shc, and p62[dok] under various stimulation conditions. Association of SHIP with p62[dok], Shc, and FcγRIIB1 was seen following FcγRIIB1-BCR coaggregation (Figure 4A). Reciprocal immunoprecipitations of both Shc and p62[dok] showed coprecipitation of ~150 kDa tyrosine-phosphorylated proteins that were reactive with a SHIP-specific antibody (Figure 4A). In addition, under these stimulation conditions, Shc coprecipitated with a small quantity of a 62 kDa tyrosine-phosphorylated protein that may be p62[dok], while p62[dok] immunoprecipitates showed coassociation of p62[dok] with a small quantity of a 52 kDa tyrosine-phosphorylated protein, possibly FcγRIIB1 or Shc. Although sequential immunoblotting using p62[dok]- and Shc-specific antibodies did not support these suggestions, anti-phosphotyrosine blotting is in our hands considerably more sensitive than specific blotting for p62[dok] or Shc. Thus, it is conceivable that ternary complexes containing SHIP, Shc, and p62[dok] exist, though they must constitute only a small minority of SHIP complexes. Major species probably consist of SHIP-FcγRIIB1, SHIP-p62[dok], and SHIP-Shc complexes. It is noteworthy that BCR-FcγRIIB1 coaggregation led to the association of p62[dok] with an unidentified ~70 kDa tyrosine-phosphorylated protein. Taken together, the data suggest that BCR aggregation alone leads to the limited formation of SHIP-Shc and SHIP-p62[dok] complexes. However, FcγRIIB1-BCR coaggregation leads to the tyrosine phosphorylation of FcγRIIB1 followed by its association with SHIP, further increasing SHIP's phosphotyrosine content as well as its subsequent association with Shc or p62[dok]. This in turn probably leads to enhanced phosphorylation of

A.

B.

C.

D.
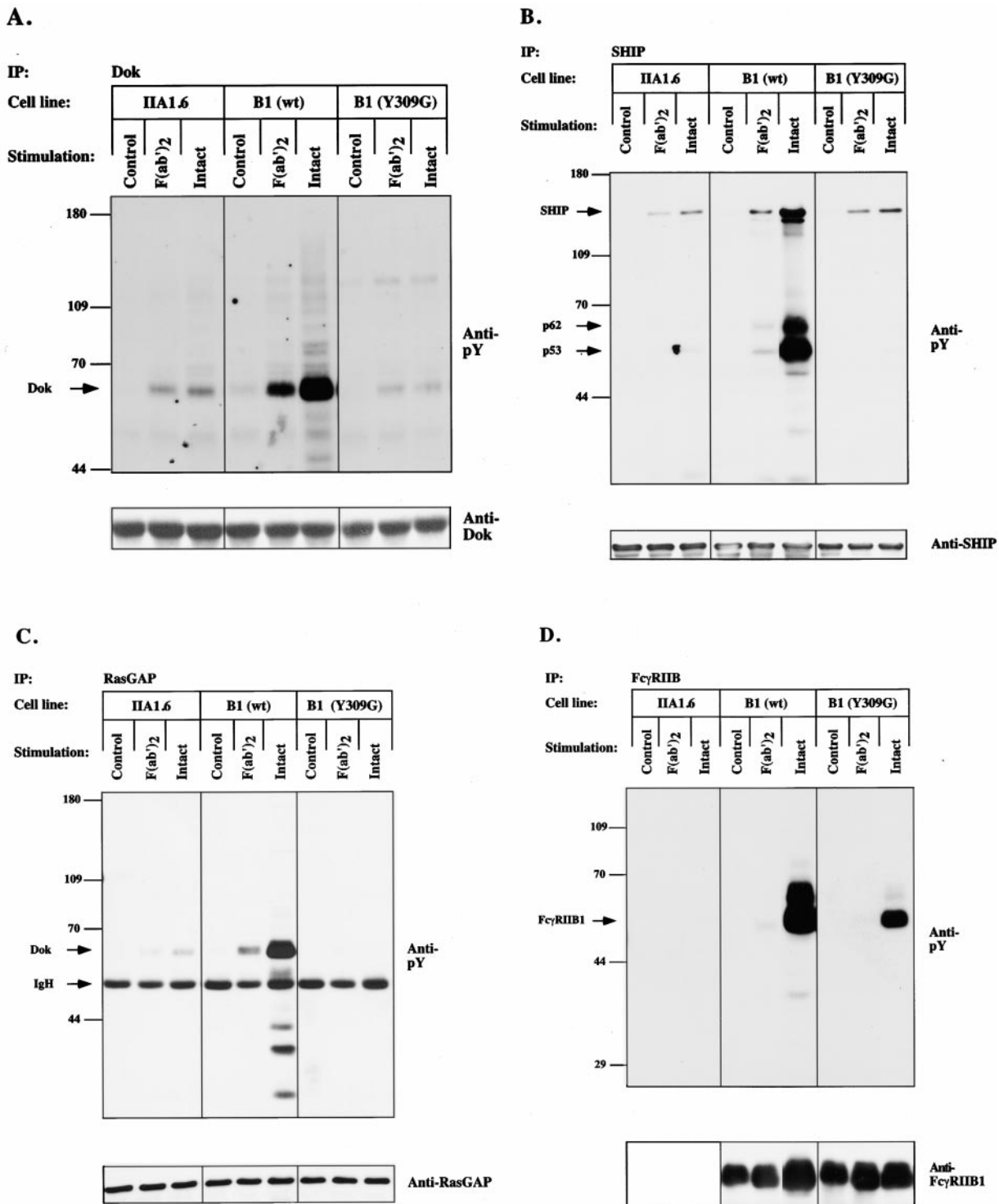
Figure 2. Increased SHIP and p62dok Tyrosine Phosphorylation and p62dok Association with RasGAP Are Dependent on the FcγRIIB1 ITIM Tyrosine

(A) FcγRIIB1 ITIM tyrosine is required for increased tyrosine phosphorylation of p62dok upon FcγRIIB1-BCR coaggregation. IIA1.6, its FcγRIIB1 transfectant (wt or the Y309G mutant) were stimulated with an IgG-specific rabbit antibody or its F(ab')₂ for 4 min at 37°C. The cells were lysed and lysates immunoprecipitated with Dok-specific antibodies followed by SDS-PAGE, electrophoretic transfer, and immunoblot analysis using an phosphotyrosine- (pY) or Dok-specific antibodies.

(B) FcγRIIB1 ITIM tyrosine is required for the increased tyrosine phosphorylation of SHIP seen upon FcγRIIB1-BCR coaggregation. Cell stimulation and lysis as described in (A). Lysates were immunoprecipitated with SHIP-specific antibodies followed by SDS-PAGE, electrophoretic transfer, and immunoblot analysis using phosphotyrosine- (pY) or SHIP-specific antibodies.

(C) The interaction between p62dok and RasGAP upon FcγRIIB1-BCR coaggregation requires FcγRIIB1 ITIM tyrosine. Cell stimulation and lysis

p62^{dok} and its association with RasGAP. Since the association of p62^{dok} with SHIP was greatly enhanced following BCR-FcγRIIB1 coaggregation (Figure 4A), we hypothesized that binding involves interactions between phosphotyrosyl residues and their respective binding domains in these molecules. In order to resolve which domains are involved in this interaction, we prepared GST fusion proteins of the N terminus of p62^{dok} (which contains its first 258 amino acid residues, including the PH + PTB domain) and of its C terminus (residues 259–482, which contains the RasGAP-binding region [Yamanashi and Baltimore, 1997]). These fusion proteins were employed to isolate binding proteins from lysates of cells stimulated by FcγRIIB1-BCR coaggregation. Results demonstrate association of tyrosine-phosphorylated SHIP with the N terminus, but not with the C terminus of p62^{dok} (Figure 4B). These observations indicate that the PH + PTB domain of p62^{dok} (Yamanashi and Baltimore, 1997) mediates its association with tyrosine-phosphorylated SHIP (see also below and Figure 5A).

Time Course of SHIP Association with p62^{dok}, Shc, and FcγRIIB1

Results shown in Figure 4A indicate that although SHIP forms binary complexes with p62^{dok}, Shc, and FcγRIIB1 upon FcγRIIB1-BCR coaggregation, ternary complexes are less frequent. This raises the issue of whether p62^{dok}, Shc, and FcγRIIB1 compete for the same pools of SHIP or bind to separate ones. To begin to address this question, we analyzed the time course of SHIP association with p62^{dok}, Shc, and FcγRIIB1 upon FcγRIIB1-BCR coaggregation. Shc, FcγRIIB1, and p62^{dok} immunoprecipitates contained some SHIP even in the absence of cell stimulation. Association of these molecules with SHIP reached a maximum with similar kinetics 2–4 min following stimulation by RAMIG (Figure 4C). Importantly, comparison of association of total SHIP versus its tyrosine-phosphorylated cohort with these molecules indicated preferential binding of Shc and p62^{dok} to tyrosine-phosphorylated SHIP, while only nonphosphorylated SHIP coprecipitated with FcγRIIB1 (Figures 4C and 4D). Considered in view of data in Figure 4A, these results suggest that at any point in time only a small fraction of FcγRIIB1-associated SHIP is phosphorylated on tyrosyl residues and able to bind Shc and p62^{dok}. Considering the amount of SHIP that is found to associate with FcγRIIB1, Shc, and p62^{dok}, together with the fact that we could not detect a signal for FcγRIIB1 in either Shc or p62^{dok} immunoprecipitates (Figure 4A), our data support a model in which SHIP dissociates from FcγRIIB1 rapidly upon its tyrosine phosphorylation and only then interacts with Shc or p62^{dok}. Alternatively, it is possible that the interaction of the tyrosine-phosphorylated form of SHIP with p62^{dok} acts to dissociate it from its interac-
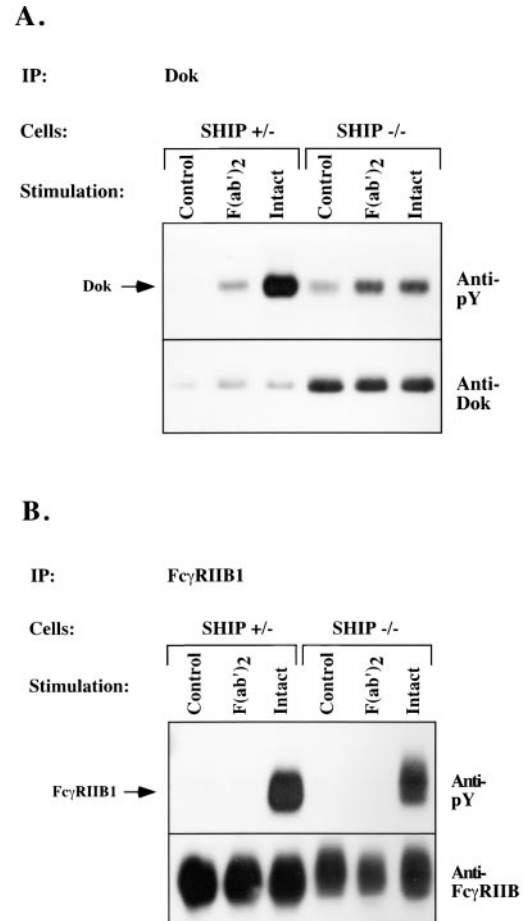

A.

IP: Dok

Cells: SHIP +/-   SHIP -/-

Stimulation: Control, F(ab')₂, Intact, Control, F(ab')₂, Intact

Dok →   Anti-pY

Anti-Dok

B.

IP: FcγRIIB1

Cells: SHIP +/-   SHIP -/-

Stimulation: Control, F(ab')₂, Intact, Control, F(ab')₂, Intact

FcγRIIB1 →   Anti-pY

Anti-FcγRIIB

Figure 3. Expression of SHIP Is Required for FcγRIIB1-BCR Coaggregation-Induced Tyrosine Phosphorylation of p62^{dok}

(A) The increase in tyrosine phosphorylation of p62^{dok} upon FcγRIIB1-BCR coaggregation is not seen in B cells from SHIP$^{-/-}$ mice. Splenic B cell blasts from SHIP$^{-/-}$ (4×10^7 cells/ml/sample) and SHIP$^{+/-}$ control littermate (2×10^7 cells/ml/sample) mice were stimulated using an IgM-specific rabbit antibody or its F(ab')₂ for 4 min at 37°C. Cells were lysed and lysates cleared as described in legend to Figure 1A. Cleared lysates were immunoprecipitated using p62^{dok}- (Dok) specific antibodies. Precipitated proteins were resolved by SDS-PAGE followed by immunoblot analysis using phosphotyrosine- (pY) or Dok-specific antibodies.
(B) Tyrosine phosphorylation of FcγRIIB1 is intact in B cells from SHIP$^{-/-}$ mice. Cell stimulation and lysis were as described in (A), with the exception that 2×10^7 cells/ml/sample were used for both SHIP$^{-/-}$ and the SHIP$^{+/-}$ control. Lysates were immunoprecipitated with an FcγRIIB1-specific rabbit antibody followed by SDS-PAGE, electrophoretic transfer, and immunoblot analysis using phosphotyrosine- (pY) or FcγRIIB1-specific antibodies.

tion with FcγRIIB1, as has been shown for Shc (Tridandapani et al., 1999). According to both of these hypotheses, phosphorylated FcγRIIB1 may act processively to

were as described in (A). Lysates were immunoprecipitated with RasGAP-specific antibodies followed by SDS-PAGE, electrophoretic transfer, and immunoblot analysis using phosphotyrosine- (pY) or RasGAP-specific antibodies.
(D) FcγRIIB1 tyrosine phosphorylation upon BCR coaggregation in the absence of FcγRIIB1 ITIM tyrosyl residue. Cell stimulation and lysis as described in (A). Lysates were immunoprecipitated with FcγRIIB1-specific rabbit antibody followed by SDS-PAGE, electrophoretic transfer, and immunoblot analysis using phosphotyrosine- (pY) or FcγRIIB1-specific antibodies.

A.

B.

C.

D.
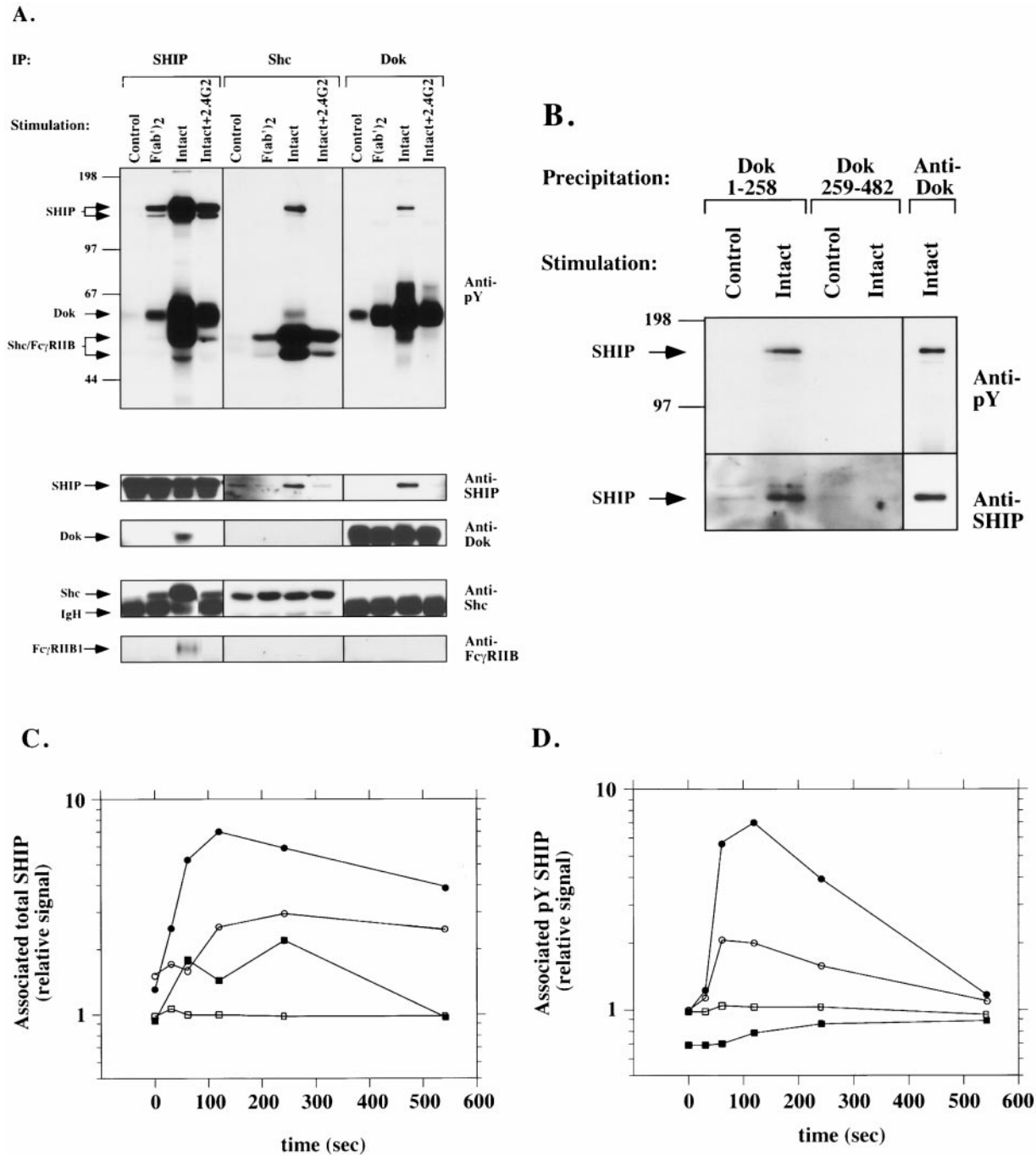
Figure 4. SHIP Associates with p62[dok], Shc, and FcγRIIB1 upon FcγRIIB1-BCR Coaggregation

(A) Association of SHIP with p62[dok], Shc, and FcγRIIB1 requires FcγRIIB1-BCR coaggregation. Untreated or 2.4G2 preincubated M12.g3r B lymphoma cells were stimulated using IgM-specific rabbit antibody (Intact) or its F(ab')$_2$ for 4 min (total) at 37°C. Preincubation with the FcγRIIB1-specific mAb (2.4G2) was performed at room temperature using 10 μg Ab/ml for 10 min. Cell lysates were precipitated with SHIP-, Shc- or p62[dok]- (Dok) specific antibodies followed by immunoblotting analysis using phosphotyrosine- (pY), SHIP-, Dok-, Shc-, or FcγRIIB1-specific antibodies (as indicated). Arrows mark position of major species.

(B) p62[dok] associates, via its PTB domain, with tyrosine-phosphorylated SHIP. Cell stimulation and lysis were as described in (A). Cleared lysates were precipitated with ~10 μg GST-Dok 1–258 or GST-Dok 259–482 fusion proteins bound to glutathione-coupled Sepharose. Precipitated proteins were resolved by SDS-PAGE followed by immunoblot analysis using phosphotyrosine- (pY) or SHIP-specific antibodies.

(C) Time course of SHIP association with p62[dok], Shc, and FcγRIIB1. M12.g3r B lymphoma cells (4 × 10^7 cells/0.5 ml/sample) were stimulated using IgM-specific rabbit antibody for the indicated duration at 37°C, followed by lysis and immunoprecipitation with p62[dok]-, FcγRIIB1-, or Shc-specific antibodies, using purified rabbit IgG as immunoprecipitation control. Precipitates were immunoblot analyzed using antibodies specific for the immunoprecipitated proteins and coprecipitated SHIP. Blots were scanned and the results analyzed as described in legend to Figure 1B (see also Experimental Procedures, Immunoblot Data Quantitation for further details). Precipitation performed using RIgG- (open squares), FcγRIIB1- (filled squares), p62[dok]- (open circles), or Shc-specific antibodies (filled circles).

generate and maintain a pool of nonreceptor-associated, tyrosine-phosphorylated SHIP, which interacts with its downstream effectors, among which are Shc and p62[dok].

Inhibition of the Erk Pathway by a Membrane-Bound Form of the RasGAP-Binding Domain of p62[dok]

To assess the role of p62[dok] in negative signaling downstream from FcγRIIB1 and SHIP, we constructed chimeric receptors containing the extracellular and transmembranal domains of FcγRIIB1 (amino acids [aa] 1–240) fused to the PH + PTB (aa 1–259) or the proline/tyrosine-rich (PTR; aa 260–482) domains of p62[dok]. These constructs, as well as full-length or tailless (tl, aa 1–240) FcγRIIB1, were expressed in the FcγRIIB1-negative B cell line IIA1.6. Stable cell lines expressing comparable levels of these constructs were isolated by cell sorting based on surface expression of FcγR. Cell populations expressing comparable levels of both BCR and FcγR were studied.

FcγRIIB-Dok 1–259 or FcγRIIB-Dok 260–482 coaggregation with BCR led to the induced tyrosine phosphorylation of ∼65 and 70 kDa proteins, respectively, consistent with the expected molecular weight of the chimeric receptor (Figure 5A). Immunoblotting analysis using the p62[dok]-specific antibody that reacted with both FcγRIIB-Dok chimeras confirmed that these phosphoproteins were the chimeric receptors (data not shown). No tyrosine-phosphorylated species were observed in FcγR immunoprecipitates from cells expressing FcγRIIB-tl under identical stimulation conditions (data not shown). Surprisingly, basal tyrosine phosphorylation of FcγRIIB-Dok 260–482 but not FcγRIIB-Dok 1–259 was evident in quiescent cells (Figure 5A), suggesting that mere plasma membrane localization of the PTR domain of p62[dok] results in its tyrosine phosphorylation. Phosphotyrosine immunoblotting analysis of FcγRIIB-Dok 1–259 immunoprecipitates revealed an additional 150 kDa species that was not present in FcγRIIB-Dok 260–482 precipitates yet reacted with SHIP-specific antibodies (Figure 5A). The tyrosine phosphorylation and association of SHIP with FcγRIIB-Dok 1–259 were enhanced upon receptor coaggregation, further supporting the notion that this domain of p62[dok] is responsible for SHIP association (see Discussion). Additionally, we observed basal association of RasGAP with FcγRIIB-Dok 260–482, which was enhanced upon BCR-FcγR coaggregation. This interaction was also observed with FcγRIIB-Dok 1–259 but was not enhanced upon BCR-FcγR coaggregation (Figure 5A). This indicates that p62[dok] association with RasGAP is mediated by the PTR domain of p62[dok] and is strongly dependent on its tyrosine phosphorylation.

To determine if p62[dok] has a direct role in inhibitory events downstream of BCR-FcγRIIB1 coaggregation, we examined the effect of FcγRIIB-Dok chimeras on Erk phosphorylation. This pathway was studied because of the inhibitory effect of FcγRIIB1 on the Ras-Erk path-
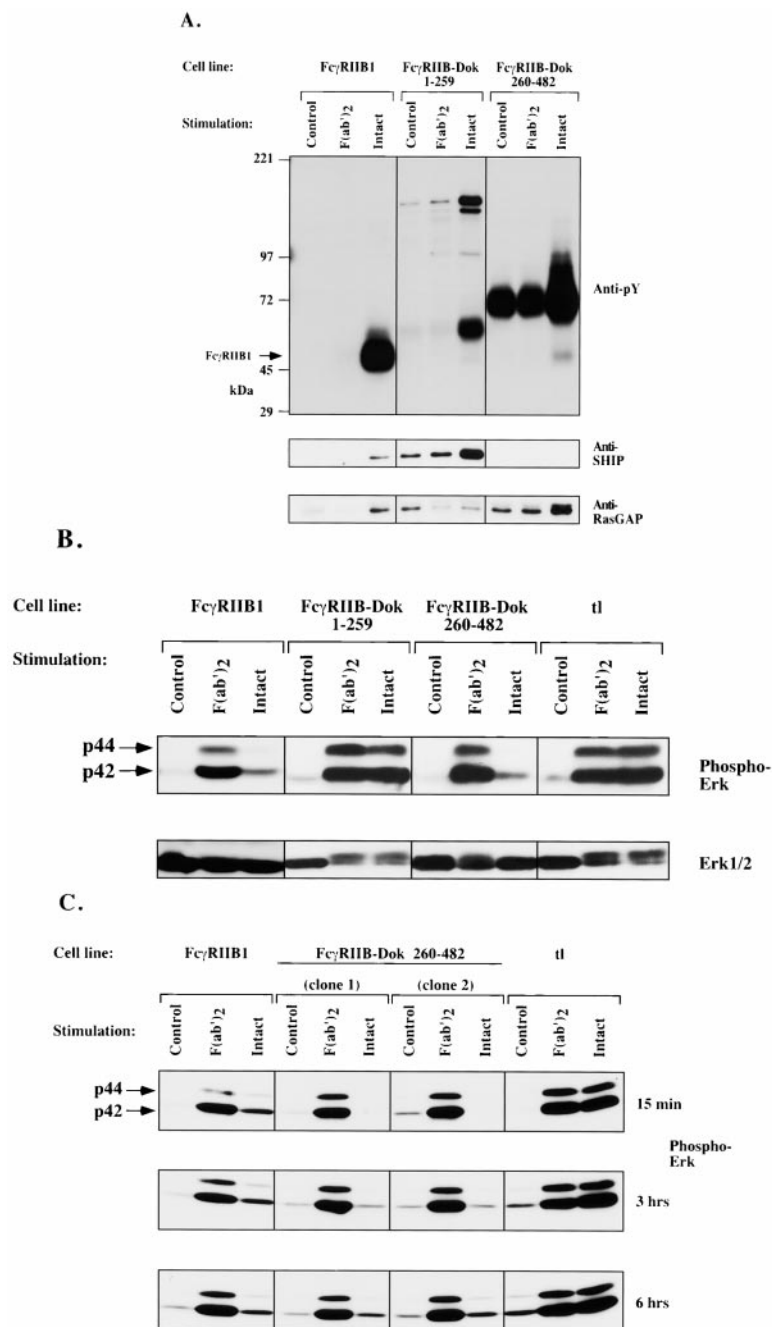
way and because RasGAP was implicated previously in Ras inhibition. Greatly reduced Erk1/2 threonine/tyrosine phosphorylation was induced in cells expressing FcγRIIB-Dok 260–482 by BCR-FcγR coaggregation (Figure 5B), and this correlated with reduced in vitro activity of Erk2 (data not shown). Importantly, this inhibition was not observed for cells expressing FcγRIIB-Dok 1–259 or those expressing FcγRIIB-tl. Furthermore, this inhibition was comparable to that observed for cells expressing FcγRIIB1 under identical stimulation conditions (Figure 5B). This inhibition was maintained for at least 6 hr poststimulation in two independent FcγRIIB-Dok 260–482 expressing cells and in cells expressing wt FcγRIIB1, but not FcγRIIB-tl (Figure 5C). The fact that inhibition of Erk1/2 phosphorylation correlated with RasGAP but not SHIP association with FcγRIIB-Dok chimeras suggests that SHIP is involved in this inhibition only via its association with p62[dok].

Discussion

The involvement of p62[dok] in control of cell differentiation and proliferation is suggested by the diversity of oncogenic kinases that induce its phosphorylation (Ellis et al., 1990). However, the signaling events that follow the tyrosine phosphorylation of p62[dok] as well as its biologic function are largely unknown. Recognition of a newly tyrosine-phosphorylated 62 kDa species in cell lysates following FcγRIIB-BCR coaggregation and previous finding that RasGAP associates with tyrosine-phosphorylated p62[dok] following BCR aggregation (Gold et al., 1993) prompted us to explore the role(s) of p62[dok] in regulation of B cell activation. Since the study by Gold et al. employed intact anti-BCR antibodies, linkage to BCR versus FcγRIIB was not distinguished. Although we observed an increase in phospho-p62[dok] upon BCR aggregation, induced p62[dok] association with RasGAP was relatively modest. However, BCR-FcγRIIB coaggregation resulted in both a dramatic increase in p62[dok] phosphorylation and association with RasGAP (Figure 1A). Furthermore, BCR aggregation with an intact anti-BCR antibody in the presence of an antibody known to block ligand binding to FcγRIIB inhibited both the increased p62[dok] tyrosine phosphorylation and association with RasGAP (Figure 4A and data not shown).

The only Fcγ receptor expressed by murine B cells is FcγRIIB (specifically, its B1 and B1′ splice variants [Latour et al., 1996]). Thus, the data demonstrate that FcγRIIB is coupled to increased p62[dok] tyrosine phosphorylation, leading to p62[dok] association with RasGAP. We further showed that receptor ITIM tyrosine is crucial for p62[dok] phosphorylation and RasGAP association (Figures 2A and 2C). Since p62[dok] does not possess a domain predicted to interact with the phosphorylated ITIM, we considered the possibility that p62[dok] interacts indirectly with FcγRIIB. Several lines of evidence support the conclusion that SHIP acts as the adaptor that links FcγRIIB

(D) Tyrosine-phosphorylated SHIP preferentially associates with Shc and p62[dok]. Cell stimulation, lysis, immunoprecipitation, and immunoblot analysis were as described in (C). Blots were also immunoblotted with phosphotyrosine-specific antibody, scanned, and the results analyzed as described in legend to Figure 1B (see also Experimental Procedures, Immunoblot Data Quantitation for further details). Legend is the same as that to (C).

A.

B.

C.

Figure 5. Dissection of p62[dok] Function using FcγRIIB Chimeras Containing Different p62[dok] Domains

(A) Tyrosine phosphorylation and association of FcγRIIB-Dok with SHIP and RasGAP. IIA1.6 cells transfected with wt FcγRIIB1 or expressing FcγRIIB-Dok chimeras were stimulated with rabbit anti-mouse IgG or its F(ab')$_2$ for 4 min at 37°C followed by lysis. Cleared lysates were immunoprecipitated with Sepharose-bound 2.4G2 beads. Precipitates were resolved by SDS-PAGE and immunoblotted using phosphotyrosine (pY)-specific antibodies (upper panel), sequentially followed by membrane stripping and immunoblotting using SHIP- or RasGAP-specific antibodies (lower panels). Results are representative of three different experiments using two different clones for every FcγRIIB chimera.
(B) FcγRIIB-Dok 260–482 coaggregation with BCR inhibits Erk phosphorylation. IIA1.6 cells transfected with wt FcγRIIB1 (3×10^7 cells/1 ml/sample) or ones expressing FcγRIIB-Dok chimeras or tailless FcγRIIB (tl) were stimulated with rabbit anti-mouse IgG or its F(ab')$_2$ for 7 min at 37°C followed by lysis. Cleared lysates were diluted in 2× SDS-PAGE sample buffer, resolved on SDS-PAGE (1×10^6 cell equivalents/lane), and immunoblotted using phosphoErk1/2-specific antibodies (upper panel), followed by membrane stripping and immunoblotting using Erk1/2-specific antibodies (lower panel). Results are representative of four different experiments using two different clones for every FcγRIIB chimera.
(C) Time course of FcγRIIB-Dok 260–482-mediated inhibition of Erk phosphorylation. IIA1.6 cells transfected with wt FcγRIIB1 (1×10^6 cells/1 ml), ones expressing the FcγRIIB-Dok 260–482 chimeras (results of two different clones are shown), or tailless FcγRIIB (tl) were stimulated with 10 μg/ml rabbit anti-mouse IgG or 12 μg/ml of its F(ab')$_2$ for 15 min to 6 hr at 37°C and 5% CO_2 followed by lysis. Cleared lysates were diluted in 2× SDS-PAGE sample buffer, resolved on SDS-PAGE (1×10^6 cell equivalents/lane), and immunoblotted using phosphoErk1/2-specific antibodies. Equivalent amounts of protein were loaded per lane as determined by sequential immunoblotting using Erk1/2-specific antibodies (data not shown).

to p62[dok]. First, a strong temporal correlation between the tyrosine phosphorylation of the two proteins was seen (Figure 1B). Second, association of p62[dok] with SHIP under negative signaling conditions was documented (Figure 4A). It was further shown that it is the PH + PTB domains of p62[dok] that associate with tyrosine-phosphorylated SHIP (Figure 4B), an interaction that probably involves NPXY motifs in SHIP. Furthermore, using B cells derived from SHIP^{−/−} mice, we showed that SHIP is required for BCR-FcγRIIB coaggregation-induced phosphorylation of p62[dok] (Figure 3A).

SHIP has previously been shown to associate via its SH2 domain (Liu et al., 1997) with some phosphorylated ITAM- (Osborne et al., 1996; Kimura et al., 1997) and

ITIM-containing receptors (D'Ambrosio et al., 1996; Fong et al., 1996) and to become tyrosine phosphorylated. This event has been shown to lead to association of SHIP with the adaptor protein Shc (Chacko et al., 1996; Tridandapani et al., 1997a). We show here that the association of Shc with SHIP occurs also in response to BCR aggregation alone but is dramatically increased upon FcγRIIB coaggregation (Figures 4A). The association of Shc with SHIP has been shown to require both the PTB domain of the former and SHIP's tyrosine phosphorylation within NPXY consensus sequences (tyrosines 917 and 1020 of murine SHIP) (Kavanaugh et al., 1995; Lamkin et al., 1997). It is likely that the observed association of the PH + PTB domain of p62[dok] with tyrosine-

phosphorylated SHIP is also mediated by phosphorylated NPXY motifs in SHIP, since it has recently been shown that FRIP—a p62^{dok} homolog present in T cells—interacts with phosphorylated NPXY motifs within the IL-4 (Nelms et al., 1998) and EGF (Jones and Dumont, 1999) receptors.

In view of the reported associations of SHIP with FcγRIIB and Shc and our observation of the association of SHIP with p62^{dok} under inhibitory signaling conditions, we analyzed the time course of these associations to determine whether they are concomitant. In addition, we studied the tyrosine phosphorylation status of associated SHIP. Our results showed that both Shc and p62^{dok} preferentially associate with the tyrosine-phosphorylated form of SHIP, while FcγRIIB associates nearly exclusively with the nonphosphorylated SHIP. Furthermore, Shc and p62^{dok} show comparable and prolonged (~10 min, Figure 4C) association kinetics with SHIP. Interestingly, the association of both Shc and p62^{dok} with SHIP is seen at later time points (>500 s) but apparently does not involve the tyrosine-phosphorylated form of SHIP (Figure 4D). It is tempting to speculate that at these time points the association of SHIP with both Shc and p62^{dok} is stabilized by SHIP SH2 domain interaction with phosphotyrosyl residues on Shc (Pradhan and Coggeshall, 1997) or p62^{dok}. Further support for the existence of such interactions for p62^{dok} is provided by the finding that BCR coaggregation with the PH + PTB domains of p62^{dok} results in massive tyrosine phosphorylation of SHIP (Figure 5A), which may be due to interaction of the latter via its SH2 domain with a phosphorylated Y(203)XXL motif in p62^{dok}.

RasGAP contributes to regulation of the balance between Ras-GDP and Ras-GTP levels by increasing the intrinsic GTPase activity of Ras (Yamanashi and Baltimore, 1997). We demonstrate here that BCR coaggregation with the PTR domain of p62^{dok}, which is involved in RasGAP binding (Figure 5A), results in inhibition of BCR-induced Erk activation (Figure 5B). Furthermore, this inhibition is maintained for at least 6 hr poststimulation (Figure 5C). Surprisingly, BCR coaggregation with the PH + PTB domains of p62^{dok}, while shown to recruit SHIP, does not result in inhibition. Thus, it is the RasGAP-binding portion of p62^{dok}, presumably acting via RasGAP, that mediates Ras/Erk pathway inhibition.

Taken together, our data suggest the following model (Figure 6) for linkage of FcγRIIB to inhibition of Ras activation: FcγRIIB-BCR coaggregation leads to the tyrosine phosphorylation of FcγRIIB on its ITIM tyrosine, which in turn leads to the recruitment and subsequent tyrosine phosphorylation of SHIP. This phosphorylation creates binding sites for the PTB domains of p62^{dok} and Shc, resulting in recruitment of these molecules to the plasma membrane. Once in the proximity of the BCR, p62^{dok} and Shc become tyrosine phosphorylated, presumably by BCR-activated Src-family kinases (Noguchi et al., 1999). Phospho-p62^{dok} recruits RasGAP, which catalyzes the intrinsic GTPase activity of Ras, converting Ras-GTP to Ras-GDP. Consequent termination of antigen receptor–mediated Ras activation prevents activation of Erk1 and 2 blocking their downstream biologic effects. Importantly, clear evidence of the biologic importance of this inhibitory pathway is provided by recent findings of Yamanashi et al. (2000), who have shown that FcγRIIB fails to inhibit BCR-mediated Erk activation

Figure 6. Model for FcγRIIB1 Inhibition of BCR-Induced Ras Activation

FcγRIIB-BCR coaggregation leads to the phosphorylation of FcγRIIB on its ITIM tyrosine, which in turn leads to the recruitment and subsequent tyrosine phosphorylation of SHIP. The latter creates a binding site for the PTB domains of p62^{dok} and Shc that acts to recruit them to the plasma membrane. Once in the proximity of the BCR, p62^{dok} becomes tyrosine phosphorylated and recruits RasGAP, which catalyzes the intrinsic GTPase activity of Ras, converting Ras-GTP to Ras-GDP, leading to inhibition of Erk activation. Full arrowheads indicate association/activation, empty arrowheads indicate transition from active to inactive forms, empty arrows indicate phosphorylation, and hammerhead indicates inhibition.

and B cell proliferation in B cells from p62^{dok} knockout mice.

In the context of other findings in this report, the observation that membrane targeting of the RasGAP-binding domain of p62^{dok} to the plasma membrane results in inhibition of BCR-mediated activation of the Ras pathway provides the first description of molecular circuitry coupling FcγRIIB to inhibition of the Ras pathway. The

more global role of p62[dok] as an inhibitory signaling intermediary awaits further research.

Experimental Procedures

Antibodies and Reagents
Purified rabbit anti-mouse immunoglobulin (H + L) (RAMIG) and anti-μ heavy chain Abs (RAMμ), as well as their F(ab')$_2$ fragments, and HRP-coupled rat anti-IgG1 and protein A were from Zymed (San Francisco, CA). Sepharose 4B-coupled RasGAP-specific mAb B4F8 was from Santa Cruz. The following Abs were also used: RasGAP-specific mAb (G12920, Transduction Labs), FcγRIIB-specific rat mAb 2.4G2 (American Type Culture Collection), Shc-specific rabbit Ab (06-203, UBI, Lake Placid, NY), pTyr-specific mAb Ab-2 (IgG1) (Calbiochem, La Jolla, CA), rabbit polyclonal Erk1- (C-16) and Erk2- (C14) specific Abs (Santa Cruz), phosphoErk-specific Ab (9101, New England Biolabs), and purified rabbit IgG (Jackson, West Grove, PA). The FcγRIIB-, SHIP-, and p62[dok]-specific Abs were generated by immunization of rabbits with the cytoplasmic tail of murine FcγRIIB1 (Nakamura and Cambier, 1998), aa residues 909–959 of murine SHIP, and full-length murine p62[dok], respectively, and purified on an antigen-coupled Sepharose 4B column. Unless otherwise stated, all other reagents were from Sigma.

DNA Constructs
GST Fusion Proteins
Murine p62[dok] cDNA was a gift from Y. Yamanashi (Yamanashi and Baltimore, 1997) and was PCR-amplified to generate p62[dok] domains using the following primer pairs: p62[dok] (1–482): 5' primer, 5'-ACGC GTCGACATGGACGGGGCTGTGATGGAGG; 3' primer, 5'-GCCGCT CGAGTCAGGTGGAACCCTCAGAC subcloned into the SalI–XhoI sites of pGEX-5X-3. The pleckstrin-homology (PH) and phosphotyrosine-binding (PTB) (1–258) and the tyrosine/proline-rich (259–482) domains of p62[dok] were PCR-amplified using the following primer pairs: 5' primer, as in p62[dok] (1–482); 3' primer, 5'-ATAAGAATGCGG CCGCGCCCACCTTTCCTTGGGCTTT, and 5' primer, 5'-CGGGATC CAGGCACAGGATATCCTC; 3' primer, as in p62[dok] (1–482) and subcloned into the SalI–NotI and BamHI–XhoI sites of pGEX-5X-3, respectively. The fusion proteins from the above constructs were purified from DH5α bacterial lysates as described earlier (Nakamura and Cambier, 1998).

FcγRIIB1 Mutants and FcγRIIB-Dok Chimeras
The generation of FcγRIIB1 Y309G mutant and its transfection into IIA1.6 cells have been described elsewhere (P. B. et al., unpublished data). Surface expression of FcγRIIB1 and its Y309G mutant were determined by flow cytometry analysis using monoclonal 2.4G2. Cell populations displaying similar surface FcγRIIB1 expression were used in experiments.

The extracellular and transmembrane domains of FcγRIIB1 (nucleotides 340–1059, which includes the signal peptide, accession no. M16367) were PCR-amplified to introduce a 5' EcoRI site followed by a Kozak sequence, and a 3' XhoI site. For the production of the tailless FcγRIIB construct (FcγRIIB-tl), a stop codon was inserted 5' of the XhoI site, and the fragment was cloned into the pMXI-egfp vector (a gift of Dr. Alice Mui, DNAX). Alternatively, for the production of FcγRIIB-Dok chimeras, the fragment without the stop codon was cloned into pMXI-egfp followed by the subcloning of PCR-amplified XhoI–NotI fragments containing p62[dok] 1–259 and p62[dok] 260–482, thus generating the respective FcγRIIB-Dok chimeras. The chimeric constructs were confirmed by sequencing. At least two different clones of each chimeric construct were used for all biochemical characterizations. The pMXI-egfp vector is a derivative of the pMX retroviral vector (Onishi et al., 1996) into which an internal ribosomal entry site (IRES) and the coding sequence for enhanced green fluorescent protein (EGFP) were cloned downstream of the polylinker. The proviral DNA is transcribed as a single polycistronic mRNA, and the resulting EGFP expression is thus a direct measure of expression of the gene of interest.

Cells and Cell Culture
The B cell lines M12.g3r (Parikh et al., 1991), A20, the FcγRIIB-deficient cell line IIA1.6 (Nakamura and Cambier, 1998), its FcγRIIB1 transfectants and FcγRIIB-Dok chimeras infectants, and Phoenix cells (Pear et al., 1993; Nolan and Shatzman, 1998) were cultured in IMDM supplemented with 5% heat-inactivated FCS (Hyclone), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C and 7% CO$_2$. All culture reagents were from GIBCO–BRL (Gaithersburg, MD). The generation of SHIP$^{-/-}$ mice has been previously described (Helgason et al., 1998). Mature B cells were purified from spleens of C57BL/6 mice using discontinuous Percoll density gradient centrifugation ($\rho > 1.066$) as previously described (Cambier et al., 1988). In the case of splenocytes obtained from SHIP$^{-/-}$ and SHIP$^{+/-}$ mice, Percoll-purified splenocytes were further enriched for B cells by culture in IMDM containing 20% FCS, 20 μg/ml LPS, 40 μg/ml dextran sulfate, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C and 7% CO$_2$ for 72 hr.

Preparation of Retrovirus and Infection of IIA1.6 Cells
High titer retroviruses carrying either FcγRIIB-tl or the FcγRIIB-Dok constructs were produced using the Phoenix packaging cell line (Pear et al., 1993; Nolan and Shatzman, 1998) (Dr. Garry P. Nolan, Stanford University, www.stanford.edu/group/nolan/NL-Homepage. html), as described previously (Nakamura and Cambier, 1998). Under these conditions, infection efficiency of IIA1.6 cells reached 90%; double-positive cells were sorted and reanalyzed prior to experimentation.

Cell Stimulation and Lysis
The cell lines A20, IIA1.6 and IIA1.6 transfectants express BCR containing IgG2a and were thus stimulated by using murine IgG (H + L)-specific rabbit Abs (and F(ab')$_2$ fragments). M12.g3r cells and mature splenic B cells express IgM and were thus stimulated by IgM-specific rabbit Abs (and F(ab')$_2$ fragments). Before stimulation, cells were washed and resuspended in IMDM at a density of 1 to 4×10^7 cells/ml. FcγRIIB was blocked with 2.4G2 (10 μg/ml for 10 min at room temperature). Cell stimulation was performed with equimolar concentrations (67 nM) of the above-described intact rabbit Abs (10 μg/ml) or their F(ab')$_2$ fragments (6.7 μg/ml) for the indicated times. For time course experiments, cells were lysed by adding an equal volume of ice-cold 2$\times$ lysis buffer (2% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.5, containing the following inhibitors: 20 mM sodium pyrophosphate, 4 mM Na$_3$VO$_4$, 20 mM NaF, 0.8 M EDTA, 2 mM PMSF, and 2 μg/ml each of aprotinin, antitrypsin, and leupeptin). For all other experiments, cells were lysed in 1$\times$ lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris, pH 7.5, containing half the concentration of all the inhibitors described above). Lysis was performed on ice for 30 min; lysates were cleared by centrifugation at 14,000 rpm and then incubated with protein A–coupled Sepharose (Amersham Pharmacia, Piscataway, NJ) to remove stimulating intact antibody.

Immunoprecipitation, SDS-PAGE, and Immunoblotting
Immunoprecipitation and SDS-PAGE were performed as described (Nakamura and Cambier, 1998) using antibody with protein A Sepharose or directly-coupled 2.4G2 Sepharose at 4°C for 1 hr. Immunoprecipitates were washed twice in ice-cold lysis buffer, boiled in reducing sample buffer, resolved on 10% polyacrylamide gels, and transferred to PVDF membranes. Membranes were blocked in TBST (0.05% Tween-20 in TBS) containing 5% BSA (Sigma) followed by incubation with the specific primary antibody in this buffer at 4°C for 1–5 hr. Membranes were then washed and incubated with HRP-conjugated secondary reagent in TBST containing 3% nonfat milk at 4°C for 1–5 hr. Finally, membranes were thoroughly washed with TBST and visualized on film using an ECL kit (NEN). For sequential immunoblotting, the membranes were stripped by incubation in a buffer containing 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris, pH 6.7, for 30 min at 50°C, followed by extensive washes with TBST, BSA blockage, and reincubation with specific primary antibodies, as described above.

Immunoblot Data Quantitation
The ECL signal was quantitated using a scanner and a densitometry program (Scan Analysis, Biosoft). In order to correct for both nonlinearity of the ECL signal and the low dynamic range of the film used, a calibration curve was generated for each experiment by serial dilution of a control sample, and film exposure time was varied to

cover the entire range of data. Following background subtraction and signal correction, the signal (e.g., phosphotyrosine) was further normalized to the amount of precipitated protein and presented as a ratio of these two signals. For the purpose of coprecipitated protein (e.g., SHIP) signal comparison between different precipitated proteins (experiments described in Figures 4C and 4D), we chose to set both the directly precipitated protein signal at t = 0 and its coprecipitated protein signal for the control immunoprecipitation (preimmune rabbit IgG) at t = 0 to 1. In this way one can compare the amount of a specific protein coprecipitating among different directly precipitated proteins and a control (rabbit IgG).

Acknowledgments

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