

RESEARCH COMMUNICATION

Role of the rasGAP-associated docking protein p62^{dok} in negative regulation of B cell receptor-mediated signaling

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Antigenic stimulation of the B-cell receptor (BCR) is a central event in the immune response. In contrast, antigen bound to IgG negatively regulates signals from the BCR by cross-linking it to the inhibitory receptor FcγRIIB. Here we show that upon cross-linking of BCR or BCR with FcγRIIB, the rasGAP-associated protein p62^{dok} is prominently tyrosine phosphorylated in a Lyn-dependent manner. Inactivation of the *dok* gene by homologous recombination has shown that upon BCR cross-linking, p62^{dok} suppresses MAP kinase and is indispensable for FcγRIIB-mediated negative regulation of cell proliferation. We propose that p62^{dok}, a downstream target of many PTKs, plays a negative role in various signaling situations.

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The p120 rasGAP-associated p62 protein was originally defined as a tyrosine-phosphorylated 62-kD protein co-immunoprecipitated with p120 rasGAP, a negative regulator of p21^{ras} (Ellis et al. 1990; White and Yenush 1998). A p62 is tyrosine phosphorylated downstream of a wide range of protein-tyrosine kinases (PTKs), such as receptors for platelet-derived growth factor (PDGF), colony stimulating factor-1 (CSF-1), insulin, insulin-like growth factor (IGF), Kit ligand, and vascular endothelial growth factor (VEGF) or oncoproteins like Bcr-Abl, a causative factor of chronic myelogenous leukemia(CML)(Carpino et al. 1997; Yamanashi and Baltimore 1997; White and Yenush 1998; Noguchi et al. 1999). A p62 is also tyrosine phosphorylated downstream of receptors coupled with cytoplasmic PTKs. We and others have independently purified a p62 from v-Abl- or Bcr-Abl-transformed hematopoietic cells and identified it as a docking protein (Dok) (Carpino et al. 1997; Yamanashi and Baltimore 1997). p62^{dok} has overall structural similarity to insulin

receptor substrate-1 (IRS-1), having both pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains in its amino-terminal portion, followed by many binding sites for SH2 domains (Carpino et al. 1997; Yamanashi and Baltimore 1997; White and Yenush 1998). IRS-1 binds to activated and autophosphorylated insulin receptor through its amino-terminal PH and PTB domains and is subsequently tyrosine phosphorylated, allowing its carboxy-terminal region to act as docking sites for SH2-containing signaling molecules such as PI-3 kinase (White and Yenush 1998). p62^{dok} appears to recruit a set of SH2-containing proteins different from those recruited by IRS-1 (Carpino et al. 1997; Yamanashi and Baltimore 1997; Noguchi et al. 1999); when tyrosine phosphorylated, it binds to rasGAP, Nck, and probably other SH2-containing proteins. Although it was recently found that ectopic overexpression of p62^{dok} in a cell line up-regulates motility of the cells (Noguchi et al. 1999), the physiological function of p62^{dok} has yet to be revealed. We have therefore generated a null mutation in the mouse *dok* gene and have studied the physiological role of p62^{dok} in primary B cells.

Results and Discussion

To address the role of p62^{dok}, we have generated a null mutation in the *dok* gene, in which the entire coding sequence of the gene was deleted by homologous recombination (Fig. 1A). *dok*^{-/-} mice did not express the *dok* protein (Fig. 1B,C). Such mice were fertile and have lived at least 1 year with normal populations of hematopoietic cells. In the equivalent cells of normal mice, however, the *dok* gene is expressed at relatively high level (data not shown). It might be that *dok*-related gene(s) such as *dok-2/frip/dok-R* can compensate for the inactivated *dok* gene (Di Cristofano et al. 1998; Jones and Dumont 1998; Nelms et al. 1998; Lock et al. 1999). p56^{dok-2} is closely related to p62^{dok} and also binds to rasGAP and Nck when tyrosine phosphorylated. However, *dok-2* gene expression is not normally detectable in B cells (Nelms et al. 1998).

In B lymphoma cell lines, a rasGAP-associated 62-kD protein is tyrosine phosphorylated upon either BCR cross-linking or BCR/FcγRIIB cocross-linking (Gold et al. 1993; Vuica et al. 1997). Thus we examined tyrosine phosphorylation of p62^{dok} in primary B cells after these treatments. When mouse splenic B cells were stimulated with the F(ab')₂ form of rabbit IgG to mouse IgM, which crosslinks only BCR (membrane IgM), p62^{dok} was tyrosine phosphorylated within 2 min after cross-linking (Fig. 2A). To our surprise, p62^{dok} was more strongly phosphorylated when B cells were stimulated with intact rabbit IgG, which cocross-links BCR and FcγRIIB. In both cases, p62^{dok} was the most prominently phosphorylated band (Fig. 2B). Tyrosine phosphorylation of p62^{dok} after these treatments was hardly detected in B cells from the *Lyn*^{-/-} mice at least during the early response (Fig. 2C) (Nishizumi et al. 1995, 1998). This suggests that

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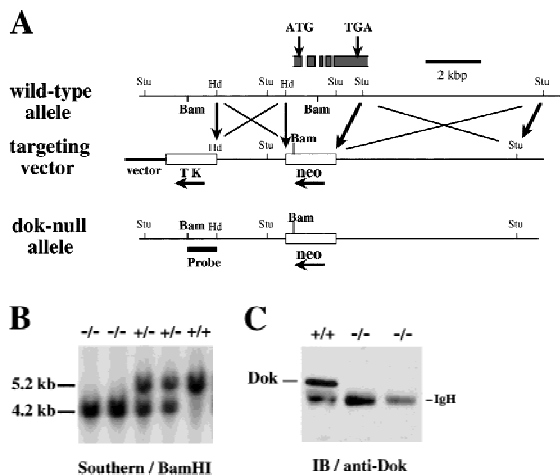


Figure 1. Generation of *dok*-null-mutant mice. (A) Restriction map of wild-type allele, targeting vector, and null allele of the *dok* gene. Positions of exons covering the entire coding region and of the initiation and stop codons are indicated by shaded boxes and arrows, respectively. The *dok* probe used for Southern blotting, as well as for thymidine kinase (*TK*) and neomycin-resistant (*neo*) genes are indicated. (*StuI* *StuI*; (*Hd*) *HindIII*; (*Bam*) *Bam*HI. (B) Southern blot analysis of genomic DNA isolated from *dok*^{+/+}, *dok*^{+/-}, and *dok*^{-/-} mice. *Bam*HI-digested genomic DNA was hybridized with the *dok* probe. Wild-type (5.2-kb) and recombinant (4.2-kb) bands are indicated. (C) Absence of Dok protein in *dok*^{-/-} mice. NP-40-soluble proteins (50 μ g) from *dok*^{+/+} and *dok*^{-/-} spleens are subjected to immunoblot analysis with mAb to p62^{dok}. Immunoglobulin heavy chain (IgH) in the lysates reactive to the secondary antibody are indicated.

p62^{dok} has a role downstream of the Src-like PTK Lyn in both BCR- and Fc γ RIIB-mediated signaling. There are several apparent target sites for Src-like PTK in p62^{dok} (Carpino et al. 1997; Yamanashi and Baltimore 1997) and coexpression of it with Lyn in 293T cells induced hyperphosphorylation of p62^{dok} (data not shown), suggesting that Lyn may phosphorylate p62^{dok}. B cells from *lyn*^{-/-} mice exhibit hyperproliferation to BCR or BCR/Fc γ RIIB stimulation, along with enhanced MAP kinase activation and Ca²⁺ influx (Wang et al. 1996; Chan et al. 1997a,b; Nishizumi et al. 1998). This hyperresponsiveness is thought to be a cause of the autoimmune disease that develops in *lyn*^{-/-} mice (Hibbs et al. 1995; Nishizumi et al. 1995). Thus, Lyn is a negative regulator of cell proliferation, MAP kinase, and Ca²⁺ influx, suggesting that p62^{dok} acts at least partially as a novel signaling molecule in pathways downstream of Lyn.

To address the function of p62^{dok}, we examined the proliferative response of splenic B cells from *dok*^{-/-} mice (Fig. 2D). Stimulation with F(ab')₂ to IgM induced proliferation of B cells irrespective of *dok* gene inactivation. However, stimulation with intact antibodies to IgM induced proliferation of B cells only from the *dok*^{-/-} mice. In addition, pretreatment with mAb 2.4G2, which blocks Fc γ RIIB, allowed intact antibody to induce proliferation of wild-type B cells. These results indicate that the pathway of negative regulation of BCR-mediated proliferation by Fc γ RIIB is dysfunctional in *dok*^{-/-} B cells at least in these experimental conditions.

Because tyrosine phosphorylation of proteins upon BCR stimulation is a critical event for subsequent cell proliferation (DeFranco 1997; Kurosaki 1997; Reth and Wienands 1997), p62^{dok} might be expected to alter the phosphorylation pattern when Fc γ RIIB is cross-linked to BCR. However, except for phosphorylation of p62^{dok} itself, overall tyrosine phosphorylation of proteins after BCR or BCR/Fc γ RIIB stimulation was not substantially altered by inactivation of the *dok* gene (Fig. 2Bb). Thus, the inhibitory effect of p62^{dok} occurs without affecting the phosphorylation of major cellular substrates for tyrosine kinases.

Upon BCR stimulation, MAP kinase activation and Ca²⁺ influx are induced and thought to be important events (DeFranco 1997; Kurosaki 1997; Reth and Wienands 1997). There are three reports on Dok family function in MAP kinase regulation. When p62^{dok} was constitutively overexpressed in a cell line, CHO-IR, which also constitutively overexpresses insulin receptor, there was a marked increase of membrane association of rasGAP upon insulin stimulation (Noguchi et al. 1999). However, the overexpression of p62^{dok} did not significantly affect MAP kinase activation upon insulin stimulation of the CHO-IR cells. On the contrary, when p56^{dok-2} was transiently overexpressed in a cell line, 32D, which also transiently overexpresses IL-2 receptor, MAP kinase activation was suppressed upon IL-2 stimulation of the cells (Nelms et al. 1998). Overexpression of p56^{dok-2} also negatively regulated EGF receptor-mediated activation of MAP kinase in COS1 cells (Jones and Dumont 1999). Therefore, we first examined if the *dok* gene inactivation in primary B cells affected MAP kinase activity upon BCR cross-linking or cocross-linking of BCR with Fc γ RIIB. Active Erk1 or Erk2 MAP kinases can be detected by their phosphorylation on both threonine and tyrosine residues in the TEY motif. We used a mAb to the active/phosphorylated form of Erk (P-Erk) to monitor its activation. Because Erk1 activation was very weak upon BCR stimulation (data not shown), we focused on Erk2. As reported previously, Erk2 activation was suppressed by costimulation of Fc γ RIIB with intact IgG (Fig. 3A; Chan et al. 1997a). However, B cells prepared from *dok*^{-/-} spleen showed higher Erk2 activation than normal cells upon BCR/Fc γ RIIB cocross-linking. In addition, Erk2 was also activated to a higher extent in the *dok*-null B cells than in normal cells even when BCR alone was cross-linked. These results indicate that p62^{dok} is a negative regulator of MAP kinase upon cross-linking of BCR or BCR with Fc γ RIIB.

Because p120 rasGAP was associated with p62^{dok} upon either BCR or BCR/Fc γ RIIB stimulation (Fig. 3B), p21^{ras}, an upstream positive regulator of MAP kinase, may be suppressed by rasGAP. However, it was reported recently that overexpression of a mutant p56^{dok-2} that could not bind to either SH2 of rasGAP in vitro could still attenuate MAP kinase (Jones and Dumont 1999). The molecular basis of Dok family suppression of MAP kinase awaits further analysis. On the other hand, even in *dok*^{-/-} B cells, Erk2 activation following BCR stimulation was still suppressed by cocross-linking it with

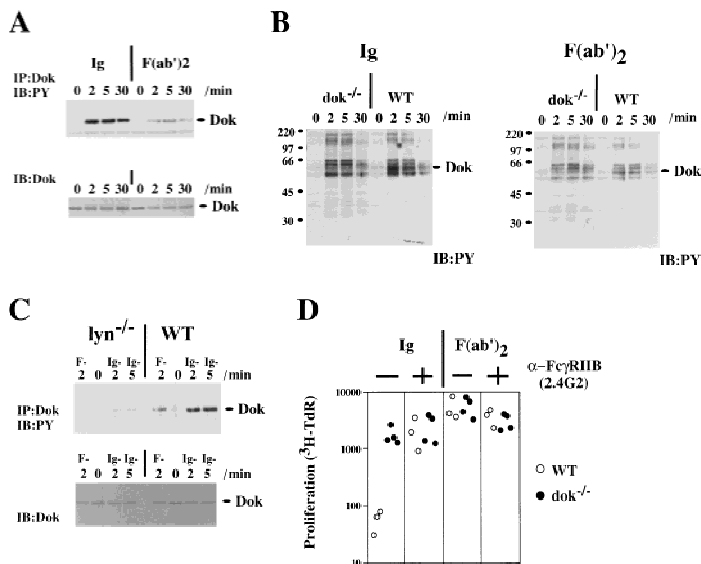


Figure 2. Lyn-dependent tyrosine phosphorylation of p62^{dok} and its role in B-cell proliferation upon BCR or BCR/FcγRIIB stimulation. (A,B) Tyrosine phosphorylation of p62^{dok}. Wild-type or *dok*^{-/-} B cells were stimulated with the intact (Ig) or F(ab')₂ form of antibody (30 μg/ml) to IgM for the indicated time. Immunoprecipitated p62^{dok} (from 6 × 10⁶ cells) (IP:Dok) or total lysate (from 2 × 10⁵ cells) of the B cells was subjected to immunoblotting with the mAb to p62^{dok} (IB:Dok) or with RC20-HRP to detect phosphotyrosine (IB:PY). Positions of p62^{dok} and standard protein markers (in kD) are indicated. (C) Lyn-dependent phosphorylation of p62^{dok}. *Lyn*^{-/-} or wild-type (C57BL/6) B cells were stimulated with the intact (Ig-) or F(ab')₂ (F-) form of antibody (30 μg/ml) to IgM for the indicated time. Immunoprecipitated p62^{dok} (from 3 × 10⁶ cells) (IP:Dok) or total lysate (from 3 × 10⁵ cells) of the B cells was subjected to immunoblotting with the mAb to p62^{dok} (IB:Dok) or with RC20-HRP (IB:PY). The position of p62^{dok} is indicated. (D) Effect of *dok* gene inactivation in B-cell proliferation. *dok*^{-/-} (●) or wild-type (○) B cells were stimulated with the intact (Ig) or F(ab')₂ form of antibody (30 μg/ml) to IgM for 2 days in the presence (+) or absence (-) of pretreatment of mAb 2.4G2 to FcγRIIB. [³H]TdR incorporation for the last 8 hr of culture was measured (counts/3 min).

FcγRIIB (Fig. 3A), suggesting that there is another molecule also suppressing MAP kinase downstream of FcγRIIB. SHIP might be the other negative regulator of MAP kinase, although that is yet controversial. (Liu et al. 1998, 1999; Okada et al. 1998).

Tyrosine phosphorylation of the ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif) of FcγRIIB and subsequent association of SHIP to the motif are critical events for FcγRIIB-mediated inhibition of Ca²⁺ influx and cell proliferation (Ono et al. 1997; Ravetch 1997;

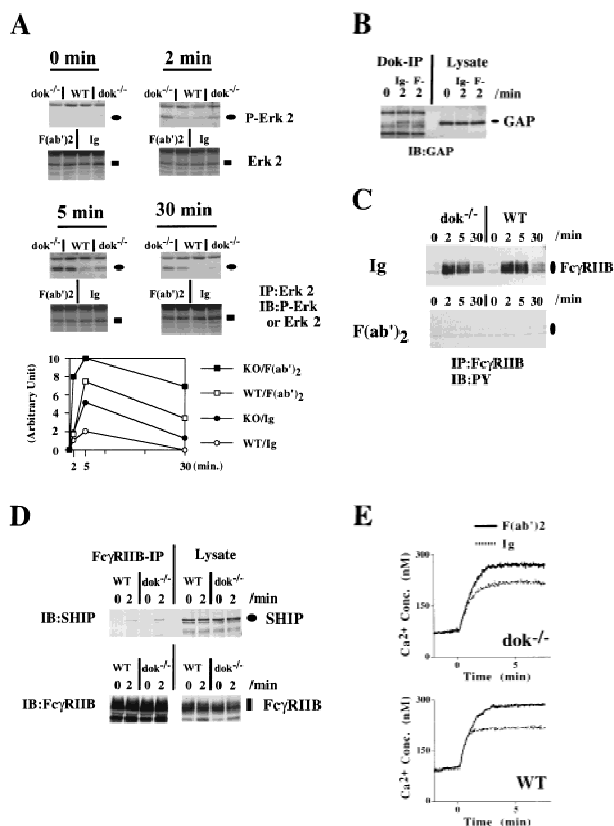


Figure 3. Role of p62^{dok} in negative regulation of MAP kinase and [Ca²⁺]_i by FcγRIIB. (A) p62^{dok}-dependent negative regulation of Erk2. *dok*^{-/-} or wild-type B cells were stimulated with the intact (Ig) or F(ab')₂ form of antibody (30 μg/ml) to IgM for the indicated time. Immunoprecipitated Erk2 (from 4 × 10⁶ cells) was subjected to immunoblotting with mAb to P-Erk2 and reprobated with antibody to Erk2. Positions of P-Erk2 (oval) and Erk2 (rectangle) are indicated (*top* panels). At each time point, the phosphorylation level of Erk2 was quantitated and normalized to the amount of immunoprecipitated Erk2 (*bottom* panel). The normalized phosphorylation level of Erk2 of *dok*^{-/-} B cells at 5 min after BCR stimulation was defined as 10 in arbitrary units. (B) Association of rasGAP to p62^{dok}. C57BL/6 B cells were stimulated with the intact (Ig-) or F(ab')₂ form (F-) of antibody (30 μg/ml) to IgM for 2 min or unstimulated (0). Proteins in anti-p62^{dok} immunoprecipitates (from 1.5 × 10⁷ cells) (Dok-IP) or total lysates (from 4 × 10⁵ cells) (lysate) were subjected to immunoblotting with the mAb to rasGAP. The position of rasGAP is indicated. (C) Tyrosine phosphorylation of FcγRIIB in *dok*^{-/-} B cells. *dok*^{-/-} or wild-type B cells were stimulated with the intact (Ig) or F(ab')₂ form of antibody (30 μg/ml) to IgM for the indicated time. Immunoprecipitated FcγRIIB (from 3 × 10⁶ cells) was subjected to immunoblotting with RC20-HRP to detect tyrosine phosphorylation. (D) Association of SHIP with FcγRIIB in *dok*^{-/-} B cells. B cells were stimulated with intact antibody to IgM for 2 min (2) or unstimulated (0). Proteins in anti-FcγRIIB immunoprecipitates (from 1 × 10⁷ cells) (FcγRIIB-IP) or total lysates (from 2 × 10⁵ cells) (lysate) were subjected to immunoblotting with antibody to SHIP or FcγRIIB. The FcγRIIB-IP blotting with antibody to FcγRIIB was visualized separately from the total lysate blotting. Positions of SHIP and FcγRIIB are indicated. (E) Negative regulation of [Ca²⁺]_i in *dok*^{-/-} B cells. *dok*^{-/-} or wild-type B cells, which were loaded with fura-2, were stimulated with the intact (dotted line) or F(ab')₂ (solid line) form of antibody (30 μg/ml) to IgM at *t* = 0 min. [Ca²⁺]_i was calculated based on the fluorescence of fura-2 bound to Ca²⁺.

Bolland et al. 1998; Coggeshall 1998; Liu et al. 1998; Scharenberg et al. 1998). Thus, p62^{dok} might be involved in the FcγRIIB/SHIP pathway. However, we found that p62^{dok} is dispensable for FcγRIIB phosphorylation and its subsequent binding to SHIP upon BCR/FcγRIIB stimulation (Fig. 3C,D). Also, increase of intracytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) following BCR cross-linking was suppressed to substantially the same extent by FcγRIIB engagement irrespective of the presence of p62^{dok} (Fig. 3E). As reported previously for wild-type B cells (Chan et al. 1997b; Nishizumi et al. 1998), intracellular released Ca²⁺, as judged by the relative [Ca²⁺]_i in the presence of EGTA in the medium returned to a basal level within 5 min after BCR or BCR/FcγRIIB stimulation in both *dok*^{-/-} and wild-type B cells (data not shown). Thus, p62^{dok} does not appear to be either a regulator or an effector of SHIP in the negative regulation of Ca²⁺ influx upon BCR/FcγRIIB cocross-linking.

FcγRIIB null mice show a relative enhancement of antibody production following both thymus-dependent (TD) and -independent (TI) antigen stimulation (Takai et al. 1996). However, *Ship*^{-/-} lymphocytes reconstituted in *Rag-1*^{-/-} mouse showed normal antibody production to vesicular stomatitis virus stimulation (Bolland et al. 1998). Therefore, SHIP is unlikely to be fully responsible for FcγRIIB-mediated negative signaling. The same was true of p62^{dok}; *dok*-null mice had normal levels of IgG₁ production to a TD antigen (Fig. 4A). In addition, *dok*-null mice showed normal levels of IgM and IgG₁ production to TI antigens (data not shown). Unexpectedly, the level of IgM production to the TD antigen in the *dok*^{-/-} mice was lower than in wild-type controls (Fig. 4A). Consistently, total IgM levels in unstimulated *dok*^{-/-} mice were also lower than in wild-type controls (Fig. 4B). Apart from this phenotype, however, p62^{dok} is unlikely to be fully responsible for FcγRIIB-mediated negative signaling. Either SHIP or p62^{dok} might be sufficient, possibly in cooperation with other molecule(s), in balancing immunoglobulin production.

Because p62^{dok} is phosphorylated downstream of mul-

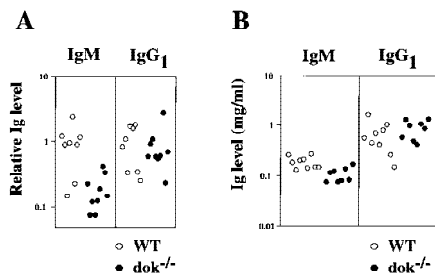


Figure 4. Low level of IgM production in *dok*^{-/-} mice. (A) IgM or IgG₁ response to TD antigen. Relative DNP-specific IgM or IgG₁ levels in sera from wild-type (○) or *dok*^{-/-} (●) mice immunized with DNP-hemocyanine were determined by ELISA, in which the mean value of immunoglobulin levels in sera from wild-type mice was defined as 1. Sera were prepared at day 7 or day 14 postimmunization for IgM and IgG₁, respectively. (B) IgM or IgG₁ concentrations (mg/ml) of preimmunization sera from wild-type (○) or *dok*^{-/-} (●) mice were determined by ELISA.

tipule growth factor receptors, and its tyrosine phosphorylation correlates with the transforming activity of Bcr-Abl (Carpino et al. 1997; Yamanashi and Baltimore 1997; Bhat et al. 1998; White and Yenush 1998), we had expected that p62^{dok} would be a positive regulator of cell proliferation. Its relative, IRS-1, has a positive role in insulin receptor signaling. However, our data show that p62^{dok}, at least in B cells, is a negative regulator of MAP kinase and cell proliferation. It appears that p62^{dok} could be important for negatively regulating the strength of signals downstream of many PTKs. In this regard, it is important that overexpression of p56^{dok-2} negatively regulates MAP kinase activity in a cell lines (Nelms et al. 1998; Jones and Dumont 1999). Because p62^{dok} and p56^{dok-2} are two major tyrosine-phosphorylated proteins in *bcr-abl*-positive primary CML cells (Carpino et al. 1997; Nelms et al. 1998), they may have a role in the establishment of the chronic phase of human CML by negatively regulating signal(s) from Bcr-Abl.

Materials and methods

Generation of *dok*-null-mutant mice

Murine *dok* genomic DNA fragments were obtained from a 129/SVJ mouse genomic library by screening with a fragment (1–448) of the murine *dok* cDNA (GenBank accession no. U78818). To construct the targeting vector, a 2.6-kb *Hind*III–*Hind*III 5' fragment and a 6.8-kb *Stu*I–*Stu*I 3' fragment were placed into the *Xho*I and *Pme*I sites of pPNT plasmid (Tybulewicz et al. 1991), respectively. This vector was linearized by digestion with *Pac*I and electroporated into J1 ES cells, which were selected with G418 and Gancyclovir. Southern blot analysis identified 5 recombinant clones with a single targeted allele among 326 clones. Targeted cells were injected into fertilized blastocysts from C57BL/6 female mice. Chimeric male mice were crossed with C57BL/6 females for germ-line transmission. Homozygotes were generated following heterozygous matings. The absence of p62^{dok} in homozygotes was confirmed by Western blotting of solubilized proteins (prepared from spleens of 8- to 11-week-old homozygous mice). All *dok*^{-/-} and control mice used were 8–11 weeks old with no splenomegaly, on a 129/C57BL/6 background or otherwise noted. All mice were maintained under specific pathogen-free conditions.

Antibodies

mAb A-3 to mouse p62^{dok} (Santa Cruz Biotechnology) was raised against its amino-terminal portion (1–276) (Yamanashi and Baltimore 1997). We confirmed that it reacts with p62^{dok} but not p56^{dok-2} by immunoblotting (data not shown). Rabbit IgG to mouse IgM was affinity purified with μ-chain, and the F(ab')₂ form of it was prepared as described (Kato et al. 1994). mAb 2.4G2 to FcγRIIB was purified with protein A–Sepharose from ascites. mAb HO-13-4 to Thy 1.2 was used in the form of ascites. Recombinant variable region of the PY20-based mAb RC20 to phosphotyrosine, conjugated with HRP (RC20–HRP), was from Transduction Laboratory. Polyclonal antibodies to Erk2 (C14), and to SHIP (N-1) were from Santa Cruz Biotechnology. Anti-mβ1 rabbit antiserum used for FcγRIIB immunoblotting was a kind gift from J.V. Ravetch (Rockefeller University, New York, NY). mAbs to phospho-Erk (E10) and p120 ras-GAP (B4F8) were from New England Biolabs and Santa Cruz Biotechnology, respectively.

Preparation of resting B cells from spleen

Splenic B cells were prepared as described previously (Kato et al. 1994). Notably, all B cells used were purified with Percoll density gradient centrifugation, which enriched small resting B cells, and the purified cells contained >95% surface Immunoglobulin-positive B cells. The same expression level of the surface FcγRIIB was confirmed on *dok*^{-/-} B cells as on wild-type controls by FACScan analysis (data not shown). With *lyn*^{-/-} and C57BL/6 B cells, they were prepared from 7-week-old mice, that did not show any splenomegaly. The *lyn*^{-/-} mice had been generated as described and backcrossed at least six generations to C57BL/6 mice (Nishizumi et al. 1995, 1998).

Immunoprecipitation and immunoblotting

B cells (1×10^7) were suspended in 200 μl of minimal essential medium (MEM) and incubated with intact or F(ab')₂ form of rabbit IgG to mouse IgM for 0, 2, 5, or 30 min at 37°C. Cells were then solubilized by adding 1 ml of 1.2× TNN buffer (1× = 50 mM Tris at pH 8.0, 150 mM NaCl, 1% NP-40, 0.2 mM sodium orthovanadate, 20 μg/ml aprotinin). The lysate was centrifuged and the supernatant was precleared by treatment with excess protein G–Sepharose. The cleared lysate was incubated with 1–2 μg of antibody to p62^{dok}, Erk2, or FcγRIIB for 1.5–3 hr, and the immune complexes were precipitated with protein G–Sepharose and washed five times with 1× TNN buffer. Proteins in the immunoprecipitates or the cleared lysates were resolved by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). Immunoblotting was performed by standard methods and visualized by chemiluminescence (NEN) or by alkaline phosphatase-based coloring (Promega). The identity of tyrosine-phosphorylated p62^{dok} in the cleared lysates (Fig. 2B) was verified by immunodepletion assay (data not shown). Quantitation of P-Erk and Erk2 immunoblotting (Fig. 3A) was performed with NIH Image software (v. 1.61).

B-cell proliferation assay

B cells (1×10^5) in 100 μl of RPMI-1640 medium, supplemented with 10% FCS, 50 μM β-mercaptoethanol, and 100 μg/ml kanamycin, were cultured in a 96-well plate in the presence of the intact or F(ab')₂ form of rabbit IgG to mouse IgM with or without pretreatment with 10 μg/ml 2.4G2 mAb to FcγRIIB, which was added to the culture at 30 min before anti-IgM stimulation. Proliferation was evaluated by pulsing with 0.25 μCi [³H]thymidine ([³H]TdR, ICN Biochemicals) for the last 8 hr of the culture. [³H]TdR incorporation was measured in a Matrix 96 system (Packard) and represented as the mean value of triplicate assays as described (Kato et al. 1994). Standard deviation was less than 5% of each readout.

[Ca²⁺]_i assay

B cells (1×10^7 cells/ml) in MEM containing 0.025% BSA were incubated with 4 μM fura-2AM at 37°C for 45 min. After washing three times, 2×10^6 fura-2-loaded B cells were suspended in 0.5 ml of the same medium and stimulated with the intact or F(ab')₂ form of rabbit IgG to mouse IgM with stirring. Emission at 500 nm was monitored under excitation of the sample at two different wavelengths (340 and 380 nm) with a CAF-110 fluorescence spectrophotometer (Japan Spectroscopic Co.). [Ca²⁺]_i was determined as described previously (Tamura and Nariuchi 1992).

Immunization and ELISA

Each 8- to 9-week-old mouse was immunized intraperitoneally

with 10 μg of dinitrophenyl (DNP)–hemocyanin in incomplete Freund's adjuvant (TD), 2 μg of DNP–Ficoll in PBS (TI), or 10 μg of trinitrophenyl (TNP)–LPS in saline (TI) at day 0. Sera were prepared at day 7 or day 14 postimmunization or preimmunization. Amounts of IgM or IgG₁ in preimmunization sera were determined by ELISA with isotype-specific antibody as described previously (Nishizumi et al. 1995, 1998). For postimmunization sera, relative amounts of IgM or IgG₁ reacting with DNP-conjugated BSA were determined by ELISA. At least seven of nine *dok*^{-/-} mice mounted IgM response to TD antigen (data not shown).

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