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Subcellular localization of Grb2 by the adaptor protein Dok-3 restricts the intensity of Ca^{2+} signaling in B cells

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Spatial and temporal modulation of intracellular Ca^{2+} fluxes controls the cellular response of B lymphocytes to antigen stimulation. Herein, we identify the hematopoietic adaptor protein Dok-3 (downstream of kinase-3) as a key component of negative feedback regulation in Ca^{2+} signaling from the B-cell antigen receptor. Dok-3 localizes at the inner leaflet of the plasma membrane and is a major substrate for activated Src family kinase Lyn. Phosphorylated Dok-3 inhibits antigen receptor-induced Ca^{2+} elevation by recruiting cytosolic Grb2, which acts at this location as a negative regulator of Bruton's tyrosine kinase. This leads to diminished activation of phospholipase C-γ2 and reduced production of soluble inositol trisphosphate. Hence, the Dok-3/Grb2 module is a membrane-associated signaling organizer, which orchestrates the interaction efficiency of Ca^{2+}-mobilizing enzymes.

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Introduction

Development, survival and activation of B lymphocytes are tightly controlled by intracellular Ca^{2+} ions, which act as second messengers in a wide range of signaling pathways (Gallo *et al*, 2006). The regulation of Ca^{2+} concentrations is a key function of the B-cell antigen receptor (BCR). BCR ligation triggers elevation of intracellular Ca^{2+} concentrations through activation of spleen tyrosine kinase Syk and subsequent phosphorylation of the adaptor protein SLP-65 (Wienands *et al*, 1998) (alternatively called BLNK, (Fu *et al*, 1998) or BASH, (Goitsuka *et al*, 1998)). Phosphorylated SLP-65 recruits Bruton's tyrosine kinase (Btk) and phospholipase C-γ2 (PLC-γ2) into a trimolecular Ca^{2+} initiation complex (Hashimoto *et al*, 1999; Ishiai *et al*, 1999a, b; Su *et al*, 1999; Chiu *et al*, 2002). This allows phosphorylation-mediated activation of PLC-γ2, which in turn hydrolyzes membrane phospholipids to yield soluble inositol trisphosphate (IP3) (Kurosaki and Tsukada, 2000). IP3 receptors are ligand-gated Ca^{2+} channels located in the membrane of the endoplasmic reticulum (ER), which stores intracellular Ca^{2+}. Hence, IP3 production causes the release of Ca^{2+} from the ER into the cytosol. The IP3-driven intracellular Ca^{2+} flux is followed by entry of Ca^{2+} from the extracellular space through weakly characterized membrane channels (Parekh and Putney, 2005; Putney, 2005). This biphasic character of the Ca^{2+} response allows shaping of the Ca^{2+} signal in the dimensions space and time, which is thought to contribute to cell fate determination during B-cell differentiation (Dolmetsch *et al*, 1997, 1998). Indeed, Koncz *et al* (2002) and Hoek *et al* (2006) reported differential Ca^{2+} signaling in BCR-activated splenic B-cell populations, which represent distinct developmental stages and are known to respond to antigen stimulation with induction of either anergy, clonal deletion or proliferation (Niiro and Clark, 2002).

Several negative regulators of the Ca^{2+} activation cascade have been described. Most prominently, the SH2 domain-containing 5′-inositol phosphatase (SHIP) interferes with membrane recruitment and concomitant activation of Btk or PLC-γ2 by disrupting the lipid binding motifs for the enzyme's pleckstrin homology (PH) domains at the inner leaflet of the plasma membrane (Ono *et al*, 1997; Bolland *et al*, 1998; Okada *et al*, 1998; Kim *et al*, 1999; Brauweiler *et al*, 2000). Also the protein tyrosine phosphatase SHP-1 and the inhibitory C-Src kinase (Csk) are implicated in the attenuation of BCR-regulated Ca^{2+} elevation and inhibition of cellular activation (Ono *et al*, 1997; Adachi *et al*, 2001). Our group has recently described the downmodulation of intra- and extracellular Ca^{2+} fluxes by the adaptor protein Grb2 (growth factor receptor-bound protein 2) (Stork *et al*, 2004). Grb2 is expressed in all cell types and throughout the B-cell lineage. It is composed of a central Src homology (SH) 2 domain flanked on either side by one SH3 domain (Lowenstein *et al*, 1992). DT40 B-cell mutants, which were rendered deficient for Grb2 expression by gene targeting (Hashimoto *et al*, 1998), but not their wild-type counterparts, showed a sustained biphasic Ca^{2+} response following BCR engagement (Stork *et al*, 2004). This raised the question how Grb2-positive B cells of the peripheral lymph organs mount a full Ca^{2+} response, which is mandatory for their antigen-mediated activation and differentiation. It turned out that stimulation-induced recruitment of cytosolic Grb2 into the lipid raft fraction of the plasma membrane prevents Ca^{2+} inhibition (Stork *et al*, 2004). Relocalization can be achieved

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by transmembrane adaptor proteins such as NTAL (non-T-cell activation linker), which upon tyrosine phosphorylation bind the SH2 domain of Grb2. NTAL expression is low in developing B cells showing a weak Ca^{2+} response, but high in mature B cells with robust Ca^{2+} elevation (Stork *et al*, 2004; Hoek *et al*, 2006). A number of additional transmembrane adaptor proteins with consensus binding sites for the Grb2 SH2 domain exist (Horejsi *et al*, 2004) and may subrogate NTAL function, for example, in NTAL-deficient mouse mutants, which possess immunocompetent B cells (Wang *et al*, 2005). The effector proteins that execute Grb2-mediated Ca^{2+} inhibition are unknown. Here we report the identification of the critical Grb2 partner for Ca^{2+} inhibition as the hematopoietic adaptor protein Dok-3 (Cong *et al*, 1999; Lemay *et al*, 2000). SH2-mediated recruitment of Grb2 to tyrosine-phosphorylated Dok-3 at the plasma membrane attenuates Btk-mediated PLC-γ2 phosphorylation independently of SHIP and Csk. Unlike positive Grb2 regulators with transmembraneous and palmitoylated polypeptide anchors, Dok-3 is tethered at the inner side of the plasma membrane through its PH domain. Hence, Dok-3 appears to direct Grb2 into a distinct membrane compartment. In this location Grb2 acts as a negative regulator of Btk, resulting in diminished PLC-γ2 activity. These findings exert a molecular basis for differential Ca^{2+} signals in B cells and moreover, directly enforce the concept that precise membrane compartmentalization of signaling elements determines positive versus negative cellular responses.

Results

Grb2 controls inducible phosphorylation of Dok-3, the main tyrosine kinase substrate protein in DT40 B cells

To assess the signaling role of Grb2 in B cells, we analyzed BCR-induced tyrosine phosphorylation in wild-type and

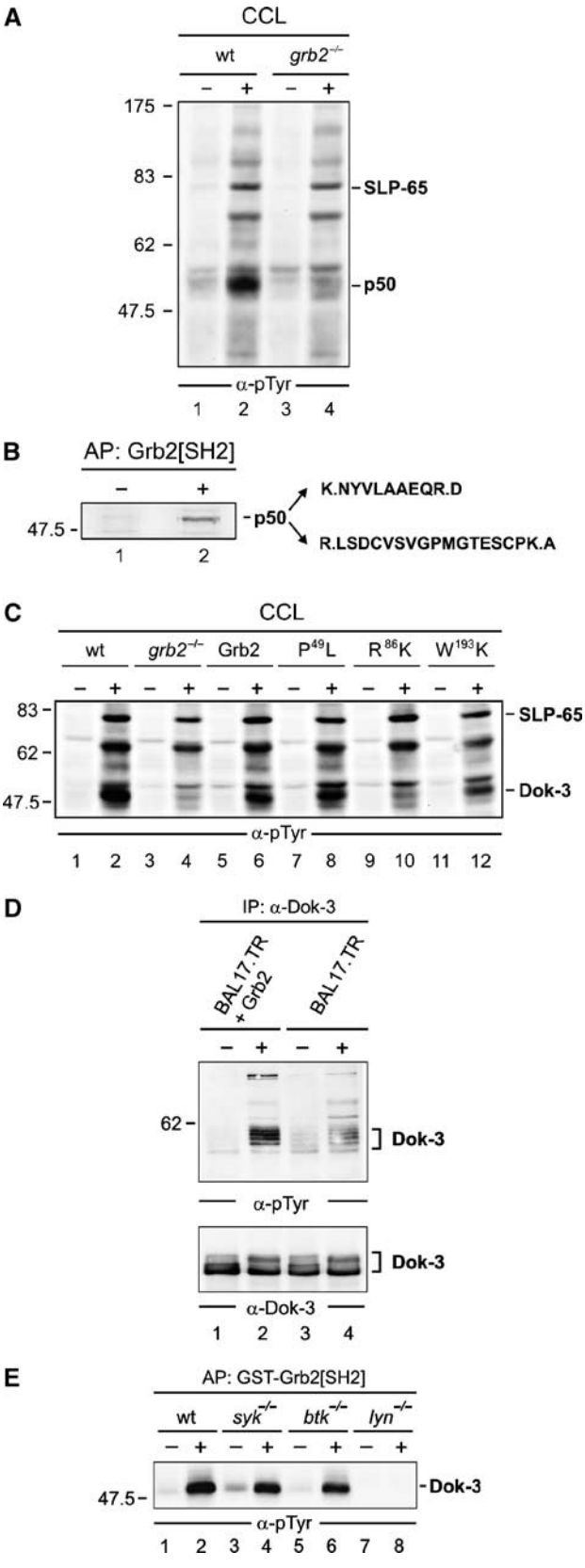

Figure 1 Grb2 controls Lyn-mediated phosphorylation of the adaptor protein Dok-3. (**A**) Wild-type (wt) and Grb2-deficient (*grb2*$^{-/-}$) DT40 cells (lanes 1, 2 and 3, 4) were left untreated (−) or stimulated through their BCRs for 3 min (+). Equal amounts of proteins from cleared cellular lysates (CCL) were analyzed by anti-phosphotyrosine (α-pTyr) immunoblotting. (**B**) The major phosphotyrosine-containing protein, p50, was affinity-purified (AP) by GST-Grb2[SH2] from stimulated DT40 cells (lane 2), silver-stained, excised, digested by trypsine and peptide products were analyzed by ESI-Trap mass spectrometry. Purified proteins from unstimulated cells served as negative control (lane 1). The obtained amino-acid sequences are shown (single-letter code) with lysine (K) and arginine (R) being inferred from trypsine cleavage specificity (indicated by dots). These sequences matched a partial chicken EST (GenBank accession number XP_427516). Full-length chicken cDNA was isolated and submitted to GenBank with the accession number EF051736 (see also Supplementary Figure S1). (**C**) Wild-type (lanes 1 and 2) and *grb2*$^{-/-}$ DT40 cells (lanes 3 and 4) reconstituted with either wild-type Grb2 (lanes 5 and 6) or Grb2 variants, in which one of the three SH domains has been inactivated by single amino-acid substitution (N-terminal SH3 domain, $P^{49}L$; SH2 domain, $R^{86}K$; C-terminal SH3 domain, $W^{193}K$; lanes 7–12), were left untreated (−) or stimulated through their BCRs (+). Equal amounts of proteins from CCL were subjected to anti-pTyr immunoblotting. To confirm equal loading, phospho-SLP-65 was detected separately by anti-SLP-65 immunoblotting (data not shown). (**D**) Murine Bal17.TR B cells, deficient for Grb2 expression, were transfected with an expression vector for Grb2 (lanes 1 and 2) or the empty vector as control (lanes 3 and 4) and left untreated (−) or stimulated through their BCRs (+). CCL were subjected to anti-Dok-3 immunoprecipitation and purified proteins were analyzed by immunoblotting with antibodies to pTyr and Dok-3 (upper and lower panels, respectively). (**E**) Resting (−) or BCR-activated (+) wild-type DT40 cells (lanes 1 and 2) or variants deficient for the protein tyrosine kinase Syk (lanes 3 and 4), Btk (lanes 5 and 6) or Lyn (lanes 7 and 8) were lysed and subjected to affinity purification with GST-Grb2[SH2]. Phosphorylated Dok-3 was detected by anti-pTyr immunoblotting. Relative molecular masses of marker proteins are indicated on the left in kDa.

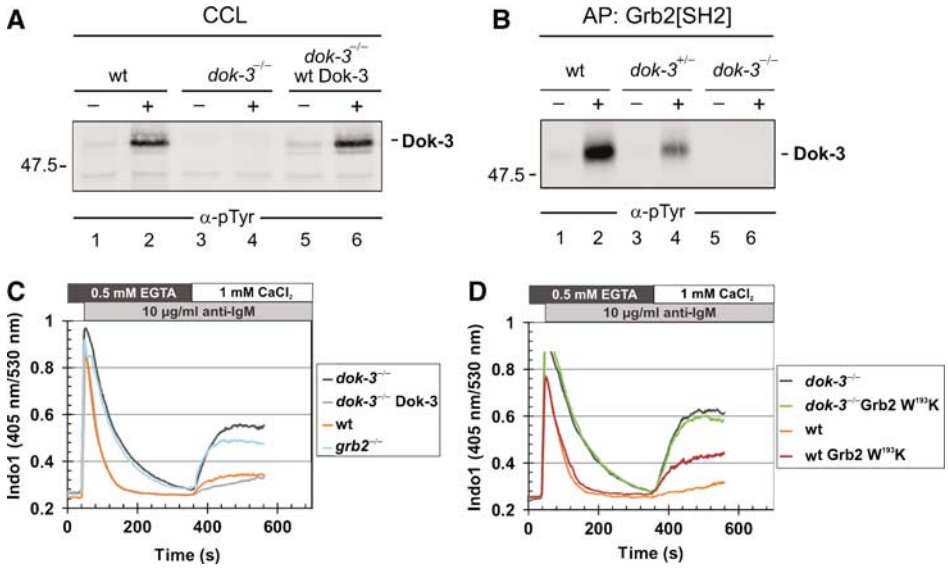

Figure 2 Gene targeting reveals a negative regulatory role of Dok-3. (**A**) Dok-3-deficient DT40 B cells were generated by targeted disruption of both *dok-3* alleles (*dok-3*$^{-/-}$, see Materials and methods for details), and absence of tyrosine-phosphorylated p50/Dok-3 in cleared cellular lysates (CCL) of resting (−) and BCR-activated (+) cells was tested by anti-pTyr immunoblotting (lanes 3 and 4). As control, wild-type DT40 and Dok-3-reconstituted *dok-3*$^{-/-}$ cells were analyzed in parallel (lanes 1 and 2 and 5 and 6, respectively). (**B**) Wild-type DT40 cells (lanes 1 and 2), heterozygous *dok-3*$^{+/-}$ (lanes 3 and 4) and homozygous *dok-3*$^{-/-}$ mutants (lanes 5 and 6) were left untreated (−) or stimulated through their BCRs (+). Cell lysates were subjected to affinity purification with the GST-Grb2[SH2] fusion protein and proteins so obtained were analyzed by anti-pTyr immunoblotting. Relative molecular mass of marker protein is indicated in (A) and (B) on the left in kDa. (**C, D**) BCR-induced intra- and extracellular Ca^{2+} mobilization of the indicated DT40 cells was recorded by flow cytometry as described in detail in Materials and methods. Briefly, cells were loaded with Indo-1 and release of intracellular Ca^{2+} was measured for 6 min in the presence of EGTA. Subsequently, extracellular Ca^{2+} was restored to 1 mM in order to monitor Ca^{2+} entry across the plasma membrane. Lines represent wild-type DT40 (orange), *dok-3*$^{-/-}$ mutants (black), Dok-3-reconstituted *dok-3*$^{-/-}$ cells (gray), *grb2*$^{-/-}$ mutants (blue) and wild-type and *dok-3*$^{-/-}$ transfectants expressing the dominant-negative $W^{193}K$ version of Grb2 (brown and green, respectively). Data are representative of at least three independent measurements.

Grb2-deficient DT40 cells. Anti-phosphotyrosine (pTyr) immunoblotting of cleared cellular lysates revealed that the main tyrosine kinase substrate protein, migrating with an apparent molecular mass of approximately 50 kDa (p50), remains almost unphosphorylated in the absence of Grb2 (Figure 1A). The association of phosphorylated p50 with the Grb2 SH2 domain (data not shown) was employed to affinity-purify large amounts of p50 from stimulated DT40 cells in order to determine the peptide profile of tryptic digestion products by mass spectrometry (Figure 1B). The obtained peptide amino-acid sequences matched to a partial chicken EST (GenBank accession number XP_427516), which shows highest homology to the murine adaptor protein downstream of kinase-3 (Dok-3). Murine Dok-3 encompasses one PH and one PTB domain at its N-terminal end, followed by consensus tyrosine phosphorylation motifs in the C-terminal half (Lemay *et al*, 2000). Our cloning of the full-length avian *dok-3* cDNA revealed that this overall structure is evolutionary conserved and that avian Dok-3 shares 68% and 62% amino-acid sequence homology to its murine and human orthologs, respectively (Supplementary Figure S1). The identity of p50 and Dok-3 was confirmed by anti-Dok-3 immunopurification (data not shown). Further reconstitution experiments with Grb2-deficient cells showed that efficient Dok-3 phosphorylation is independent of the N-terminal SH3 domain of Grb2, but requires the SH2 and C-terminal SH3 domains (Figure 1C, lanes 7–12). Similar to avian Dok-3, efficient tyrosine phosphorylation of murine Dok-3 is also dependent on Grb2 expression, as revealed by our analysis of Grb2-deficient mouse B-cell line Bal-17.TR and its Grb2-reconstituted transfectants (Figure 1D). As shown in Figure 1E, inducible tyrosine phosphorylation of Dok-3 is detectable in the absence of Syk (lanes 3 and 4) and Btk (lanes 5 and 6), but requires expression of the Src family kinase Lyn (lanes 7 and 8). Collectively, these data identify the intracellular adaptor protein Dok-3 as a major substrate of Src family kinases in activated B cells. The efficiency of Dok-3 tyrosine phosphorylation is, however, critically dependent on the additional presence of Grb2, which we have previously described as a negative regulator of BCR-induced Ca^{2+} mobilization.

Dok-3 is a negative regulator of BCR-induced Ca^{2+} mobilization

To functionally characterize Dok-3, we generated a Dok-3-deficient DT40 variant by gene targeting (see Materials and methods and Supplementary Figure S2A for details). Successful inactivation of *dok-3* alleles and ablation of protein expression was confirmed by genomic PCR analysis (Supplementary Figure S2B) and anti-pTyr immunoblotting of cleared cellular lysates, Grb2[SH2]-purified proteins and anti-Dok-3-immunoprecipitates (Figure 2A and B; Supplementary Figure S2C). Note that Dok-3 tyrosine phosphorylation is considerably reduced in heterozygous *dok-3*$^{+/-}$ cells (Figure 2B, lanes 1–4).

Given the reported role of Grb2 for BCR-induced Ca^{2+} signaling (Stork *et al*, 2004), we next tested this response in various DT40 cell lines, which are positive or negative for Dok-3 or Grb2 (Figure 2C). In marked contrast to wild-type DT40 cells, Dok-3-deficient cells show a biphasic Ca^{2+} profile, which is almost identical to that of Grb2-deficient cells (Figure 2C, orange, black and blue lines). The monophasic Ca^{2+} response of wild-type DT40 cells, which is characteristic for B cells with an immature phenotype (Koncz *et al*, 2002; Stork *et al*, 2004; Hoek *et al*, 2006), was

restored in the Dok-3 mutant cells upon reconstitution with wild-type Dok-3 (gray line). These results show that similar to Grb2, Dok-3 is a negative regulatory element of BCR-induced Ca^{2+} mobilization. Moreover, both adaptor proteins appear to function in a common signaling pathway. To further confirm the latter notion, we employed a dominant-negative Grb2 mutant protein, which harbors an inactivated C-terminal SH3 domain ($W^{193}K$). Expression of Grb2 $W^{193}K$ in DT40 cells overwrote the inhibitory function of endogenous wild-type Grb2 and allowed extracellular Ca^{2+} influx (Figure 2D, brown and orange lines). In marked contrast, expression of the Grb2 $W^{193}K$ protein in Dok-3-deficient DT40 cells had no effect on the Ca^{2+} profile (black and green lines), which strongly suggests that Dok-3 and Grb2 build a functional unit to attenuate BCR-induced Ca^{2+} mobilization.

Plasma membrane tethering and association to Grb2 are sufficient for Dok-3 to inhibit Ca^{2+} signaling

To elucidate the structural requirements of Dok-3 for Ca^{2+} inhibition, we expressed a series of HA-tagged Dok-3 mutants (see Figure 3A) in *dok-3*$^{-/-}$ cells. Inactivation of the PTB domain ($R^{197}A$) had no effect on the ability of Dok-3 to

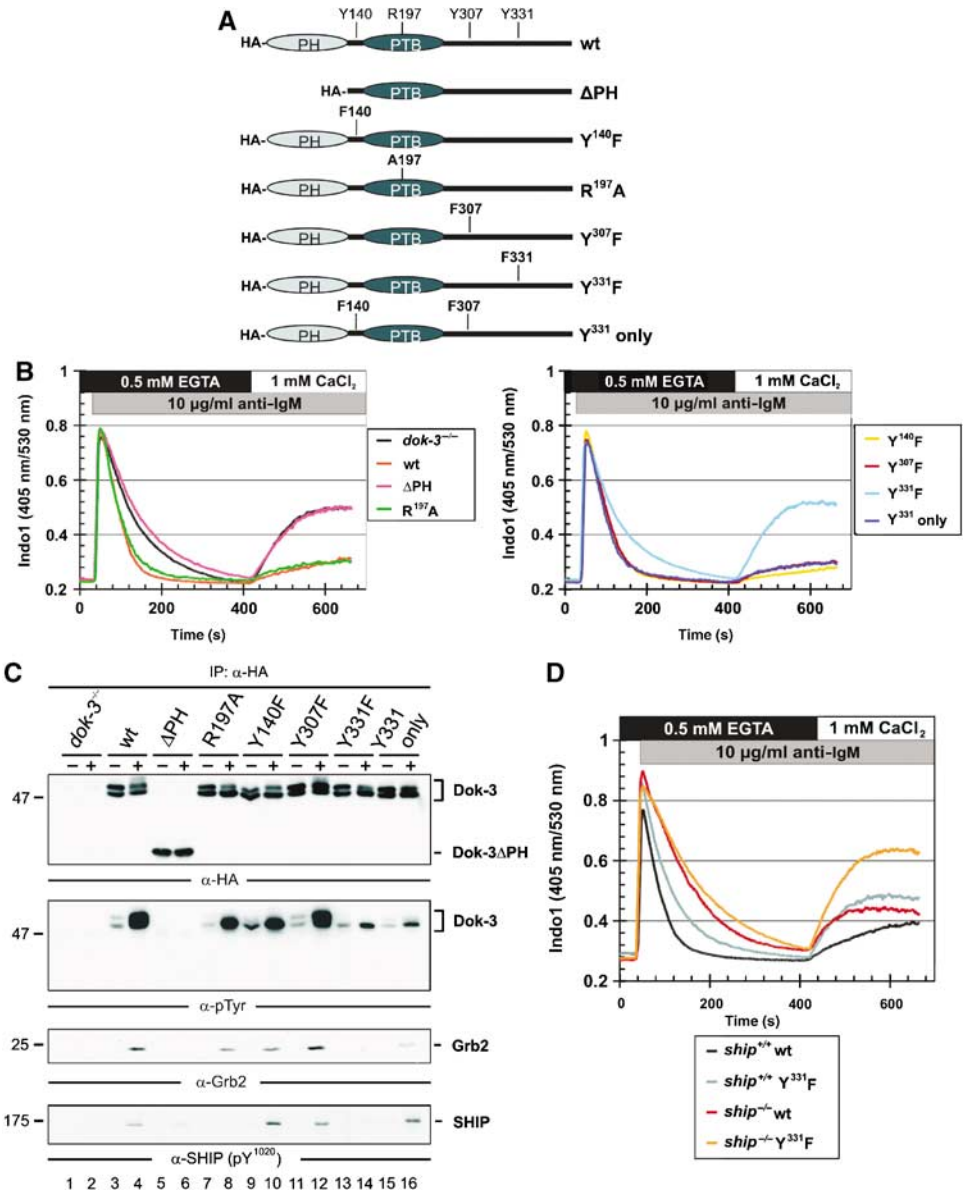

Figure 3 Dok-3 and Grb2 build a functional unit, that inhibits Ca^{2+} flux independent of SHIP. (**A**) Schematic representation of expression constructs encoding HA-tagged versions of wild-type Dok-3, a PH domain deletion mutant (ΔPH) or mutants encompassing amino-acid exchanges depicted in single-letter code. (**B**) Expression vectors were introduced by retroviral transduction in *dok-3*$^{-/-}$ mutants and BCR-induced Ca^{2+} mobilization of the transfectants was measured by flow cytometry, as described in the legend to Figure 2. Wild-type DT40 cells and empty vector transfectants of *dok-3*$^{-/-}$ mutants served as control (see inlay for color code). (**C**) Wild-type and DT40 variants described in (B) were left untreated (−) or BCR-activated (+) and lysates were subjected to anti-HA immunoprecipitation. Expression and tyrosine phosphorylation of Dok-3 proteins, as well as their association to Grb2 and SHIP, were detected by sequential immunoblotting with antibodies to HA, pTyr, Grb2 and SHIP (upper to lower panels, respectively). (**D**) BCR-induced Ca^{2+} fluxes were analyzed as described in the legend to Figure 2 in SHIP-deficient DT40 cells (*ship*$^{-/-}$, brown) and *ship*$^{-/-}$ transfectants expressing a Dok-3 $Y^{331}F$ variant that counteracts Ca^{2+} inhibition by endogenous wild-type Dok-3 (orange). As control, parental DT40 cells, which are positive for endogenous SHIP and Dok-3 (black), and the Dok-3 $Y^{331}F$ transfectants (gray) were analyzed in parallel, demonstrating the dominant-negative function of Dok-3 $Y^{331}F$.

prevent extracellular Ca^{2+} entry (Figure 3B, left panel, green and orange lines). Deletion of the PH domain (ΔPH) abolished Dok-3-mediated Ca^{2+} inhibition, which resulted in a biphasic response that was indistinguishable from that observed in cells with no Dok-3 expression (left panel, red and black lines). Single and dual Y-to-F amino-acid substitutions revealed that among the consensus tyrosine phosphorylation motifs of Dok-3, only that at Y^{331} is essential and sufficient for Ca^{2+} inhibition, whereas those at Y^{140} and Y^{307} are dispensable (right panel). Immunoprecipitation with anti-HA antibodies and subsequent immunoblot analysis showed that wild-type and mutant Dok-3 proteins are expressed by the transfectants at similar levels (Figure 3C, upper panel). This setting was also used to investigate the tyrosine phosphorylation status of the various Dok-3 proteins by anti-pTyr immunoblotting (Figure 3C, second panel). Inducible phosphorylation was easily and at almost identical levels detectable for wild-type Dok-3 (lanes 3 and 4) and Dok-3 mutants $R^{197}A$ and $Y^{140}F$ and $Y^{307}F$ (lanes 7–12), which all promoted the same biphasic Ca^{2+} profile (see above). In marked contrast, the Dok-3ΔPH protein did not become phosphorylated (lanes 5 and 6), and that of the $Y^{331}F$ mutant was strongly diminished (lanes 13 and 14). Both of these Dok-3 mutants were unable to support Ca^{2+} inhibition (see above). A strongly reduced tyrosine phosphorylation was also observed for the Y^{331} only protein (lanes 15 and 16) that, however, was fully capable of attenuating BCR-induced Ca^{2+} flux (see above). Collectively, we conclude that PH domain-mediated plasma membrane localization of Dok-3 is a requisite for Ca^{2+} inhibition, which itself is tightly associated with Dok-3 tyrosine phosphorylation. The latter event *per se* appears to be necessary but not sufficient for Ca^{2+} regulation. Rather, specific phosphorylation at Y^{331} is the second key element of Dok-3-mediated Ca^{2+} regulation.

Phosphorylation of Y^{331} creates a consensus binding site for the Grb2 SH2 domain. Indeed, the inducible association of Dok-3 with Grb2 was lost in cells expressing the $Y^{331}F$ mutant of Dok-3 (Figure 3C, third panel, lanes 13 and 14). Also the signaling-inactive ΔPH domain mutant did not co-immunoprecipitate with Grb2 (Figure 3C, third panel, lanes 5 and 6). For all other Dok-3 mutants, which retained their inhibitory capacity, BCR-induced complex formation with Grb2 was preserved (lanes 7–12). Hence, the biochemical property of inducible Grb2 association directly correlates with the functional ability of Dok-3 to downmodulate Ca^{2+} signals. This further demonstrates that Dok-3 and Grb2 together constitute a Ca^{2+}-regulating signaling module.

Dok-3 has been previously reported to associate with SHIP and Csk via the PTB domain and phospho-Y^{307}, respectively (Lemay *et al*, 2000; Robson *et al*, 2004). Indeed, the $R^{197}A$ amino-acid exchange in the PTB domain of Dok-3 abolished SHIP binding, which moreover appeared to require specific phosphorylation at Y^{331} (Figure 3C, lower panel). SHIP, however, is a well-known inhibitor of BCR-induced Ca^{2+} elevation, and it was therefore unexpected that disruption of the Dok-3/SHIP complex had no effect on the Ca^{2+} response. Hence, we wanted to confirm the missing role of SHIP with a second experimental setting. For this purpose, we employed the $Y^{331}F$ mutant of Dok-3, which counteracted Ca^{2+} inhibition by wild-type Dok-3, and when expressed in DT40 cells allowed for entry of extracellular Ca^{2+} (Figure 3D, black and gray lines). We reasoned that if Dok-3 controls Ca^{2+} through SHIP, expression of the $Y^{331}F$ dominant-negative version in SHIP-deficient cells should have no effect on the extracellular Ca^{2+} influx observed in these cells (Figure 3D, brown line). However, and consistent with our mutational analysis described above, expression of the $Y^{331}F$ mutant in *ship*$^{-/-}$ cells strongly augmented intra- and extracellular Ca^{2+} mobilization (orange line). This result demonstrates that inhibition of Ca^{2+} signals by endogenous wild-type Dok-3 is independent of SHIP expression. Final proof that SHIP is not a major downstream effector of Dok-3 came from the biochemical analysis of SHIP itself and its downstream target, the kinase Akt (alternatively called PKB). Neither phosphorylation of SHIP nor of Akt/PKB was drastically altered in the absence of Dok-3 expression (Supplementary Figure S3A). Similar to SHIP, also the catalytic activity of the Dok-3 binding partner Csk appeared unaltered in *dok-3*$^{-/-}$ cells (Supplementary Figure S3B), which further supports our mutational analysis. In summary, SHIP and Csk are both dispensable for Dok-3-mediated regulation of Ca^{2+}, demonstrating that these proteins do not function together in a common Ca^{2+} signaling pathway.

PLC-γ2 is a target of Dok-3

In search for an enzymatic activity that is under the control of Dok-3, we focused on PLC-γ2. First, we tested the overall tyrosine phosphorylation status of PLC-γ2, which appeared to be very similar in the presence and absence of Dok-3 (Figure 4A). Using a site-specific antibody that detects phosphorylation of Y^{759} (in human PLC-γ2), we observed drastic differences between Dok-3-positive and Dok-3-negative cells (Figure 4B). The kinetic and extent of PLC-γ2 phosphorylation at this specific residue was substantially upregulated in *dok-3*$^{-/-}$ cells (lanes 5–8) compared with wild-type parental cells (lanes 1–4) or Dok-3-reconstituted transfectants (lanes 9–12). Phosphorylation of Y^{759} is known to be dependent on Btk and directly correlates with the enzymatic activity of PLC-γ2 (Humphries *et al*, 2004; Kim *et al*, 2004). Indeed, the hydrolysis of membrane phospholipids was more rapid and efficient in *dok-3*$^{-/-}$ cells than in reconstituted transfectants, as shown by monitoring the intracellular levels of the PLC-γ2 product IP3 (Figure 4C, left panel). The same was also true for *grb2*$^{-/-}$ cells (Figure 4C, right panel). These data identify PLC-γ2 as an effector protein of the Dok-3/Grb2 signaling module.

Membrane-bound Dok-3 controls BCR-induced relocalization of Grb2

Stimulation-dependent plasma membrane anchoring is a critical event for PLC-γ2 function (Nishida *et al*, 2003). This led us to investigate the *in vivo* subcellular localization of Dok-3 and Grb2 in resting and BCR-activated cells by confocal laser scanning microscopy (Figure 5). Dok-3-deficient DT40 cells were reconstituted with GFP-tagged versions of either wild-type or mutant Dok-3. Wild-type Dok-3 was constitutively and almost exclusively localized at the plasma membrane (Figure 5A, upper row). Nonetheless, BCR activation appeared to induce intra-membraneous relocalization of Dok-3, as indicated by the shift from uniform plasma membrane staining in resting cells to dotted fluorescence signals in stimulated cells. Membrane tethering was completely lost for the ΔPH mutant of Dok-3, which was homogeneously distributed in the cytoplasm of the cells (Figure 5A, middle

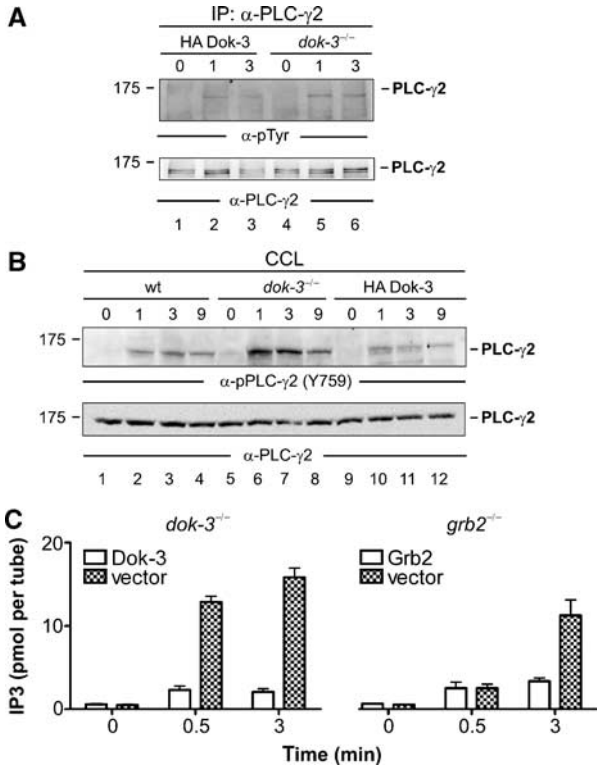

Figure 4 The Dok-3/Grb2 module attenuates PLC-γ2 activity. (**A**) Dok-3-deficient DT40 mutants (lanes 4–6) and reconstituted cells expressing HA-tagged wild-type Dok-3 (lanes 1–3) were left untreated (0) or stimulated through their BCRs for the indicated times (min). Lysates were subjected to anti-PLC-γ2 immunopurification and proteins obtained were analyzed by anti-pTyr and anti-PLC-γ2 immunoblotting (upper and lower panels, respectively). (**B**) Parental DT40 cells (lanes 1–4), *dok-3*$^{-/-}$ mutants (lanes 5–8) and HA-Dok-3-reconstituted transfectants (lanes 9–12) were left untreated (0) or stimulated through their BCRs for the indicated times (min). Cleared cellular lysates (CCL) were subjected to immunoblot analysis with antibodies that specifically detect PLC-γ2 phosphorylation at the Btk-dependent phospho-acceptor site corresponding to Y^{759} in human PLC-γ2 (upper panel). Equal protein loading was confirmed by reprobing the membrane with anti-PLC-γ2 antibodies (lower panel). Relative molecular mass of marker protein is indicated in (A) and (B) on the left in kDa. (**C**) DT40 mutant cells deficient for either Dok-3 (left panel) or Grb2 (right panel) and the empty vector control transfectants (open and filled bars, respectively) were left untreated (0) or BCR-activated for 0.5 or 3 min. IP3 levels in these cells were measured using a competitive binding assay with radiolabelled IP3-binding proteins. Error bars represent s.e.m. of three independent experiments with double preparation.

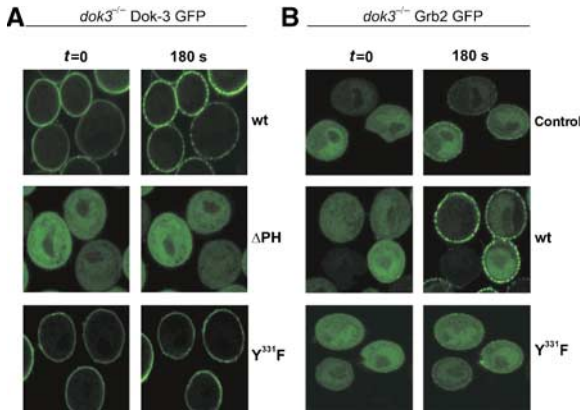

Figure 5 Dok-3 is permanently localized at the plasma membrane and is essential for stimulation-dependent recruitment of Grb2. (**A**) Dok-3-deficient DT40 mutants were transfected with expression constructs encoding fusion proteins between the green fluorescence protein (GFP) at the C terminus and either wild-type Dok-3 (upper row), Dok-3ΔPH (middle row) or Dok-3 $Y^{331}F$ (lower row) at the N terminus. Subcellular localization of Dok-3/GFP fusion proteins in resting ($t = 0$) or BCR-activated (180 s) cells (left and right images) was visualized by confocal laser scanning microscopy. (**B**) *Dok-3*$^{-/-}$ DT40 cells expressing a Grb2/GFP fusion protein were transfected with either empty control vector (upper row) or expression vectors encoding wild-type or $Y^{331}F$ Dok-3 mutants (middle and lower rows). Subcellular Grb2 localization was analyzed as in (A). The Ca^{2+} signaling function of GFP fusion proteins was tested separately (data not shown).

row). The $Y^{331}F$ mutant of Dok-3 behaved like the wild-type protein (Figure 5A, lower row).

Next, we assessed the role of Dok-3 for subcellular localization of Grb2. Expression of GFP-tagged Grb2 in unstimulated *dok-3*$^{-/-}$ cells resulted in fluorescence staining of the cytoplasm but not the plasma membrane (Figure 5B, upper left). Following BCR activation, minute amounts of Grb2 could be detected at the plasma membrane, but the overall staining pattern remained unchanged (Figure 5B, upper right). In marked contrast, in the presence of wild-type Dok-3, the vast majority of Grb2 translocated to the plasma membrane in a stimulation-dependent manner (Figure 5B, middle row). Expression of the $Y^{331}F$ mutant of Dok-3 did not support this relocalization of Grb2 (Figure 5B, lower row).

Altogether, our *in vivo* imaging reveals that plasma membrane-bound Dok-3 recruits majority of cytosolic Grb2 in BCR-activated DT40 cells most likely by phospho-Y^{331}/SH2-interaction. Notably, the PH domain-anchored Dok-3 itself undergoes a BCR-triggered clustering within the plasma membrane.

Dok-3 homo-oligomerizes upon BCR activation

To further dissect possible clustering processes of membrane-bound Dok-3, we coexpressed GFP-tagged Dok-3 with HA-tagged versions of wild-type or mutant Dok-3 proteins. Subsequently, their ability to form higher aggregates in resting and BCR-activated cells was biochemically investigated by co-immunoprecipitation experiments in which anti-HA-purified proteins were analyzed by Western blotting with antibodies to GFP and HA (Figure 6, upper and lower panels, respectively). In the analysis of *dok-3*$^{-/-}$ cells, parental cells served as specificity control (lanes 1 and 2). Dok-3-GFP coprecipitated with wild-type Dok-3 from both unstimulated and stimulated cells, but the efficiency strongly increased upon BCR activation (lanes 3 and 4). The stimulation-dependent, but not the constitutive association between Dok-3 proteins, was strongly diminished for those mutants that either lack the PH domain (ΔPH, lanes 5 and 6), possess an inactivated PTB domain ($R^{197}A$, lanes 7 and 8) or cannot be phosphorylated at Y^{140} ($Y^{140}F$, lanes 9 and 10). Phosphorylation of the Grb2-binding site Y^{331} appeared to be dispensable ($Y^{331}F$, lanes 11 and 12). These data demonstrate a homotypic and inducible aggregation of Dok-3 proteins. For BCR-triggered upregulation of this interaction, Dok-3 must be localized at the plasma membrane, which allows for phosphorylation at Y^{140} (see also Figure 3C, lanes 5 and 6). Most likely, phospho-Y^{140} is then intermolecularly bound by the PTB domain of a neighboring Dok-3 molecule. The

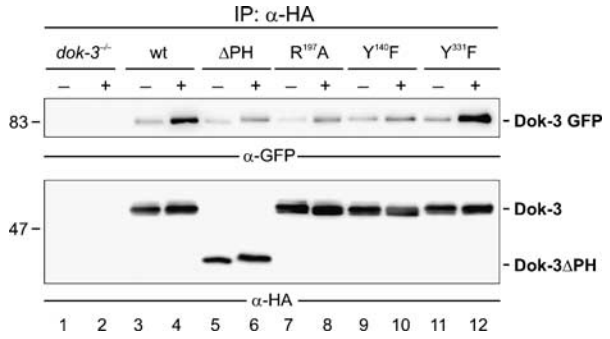

Figure 6 Dok-3 undergoes stimulation-dependent homo-oligomerization. *Dok-3*$^{-/-}$ mutant cells were reconstituted with Dok-3/GFP and subsequently transfected with empty control vector (lanes 1 and 2) or expression constructs for either HA-tagged wild-type Dok-3 (lanes 3 and 4) or the indicated HA-tagged Dok-3 variants (lanes 5–12; for details, see Figure 3A). Lysates were subjected to anti-HA immunoprecipitation. Proteins obtained were analyzed by immunoblotting with antibodies to GFP (upper panel) and HA peptide tag (lower panel). Relative molecular masses of marker proteins are indicated on the left in kDa.

subsequently induced multimerization cascade is independent of Grb2 recruitment and vice versa, Grb2 binding and concomitant Ca^{2+} inhibition is independent of the Dok-3 oligomers (see also Figure 3B and C). Hence, oligomerization of and Ca^{2+} inhibition by Dok-3 proteins are two separate and functionally independent processes.

Discussion

Intracellular elevation of Ca^{2+} in B cells is a tightly regulated process, which involves positive and negative control elements. Interfering with proper regulation of Ca^{2+} signaling is known to perturb humoral immune responses in mouse and man (Wienands, 2000). Herein, we have elucidated mechanistic details of Ca^{2+} inhibition through the adaptor proteins Grb2 and Dok-3. Several lines of evidence obtained by biochemical, genetic and imaging techniques show that following BCR activation, Grb2 and Dok-3 constitute a functional unit in a negative feedback control loop at the plasma membrane. First, following Lyn-mediated phosphorylation, Dok-3 associates with Grb2 by virtue of the Grb2 SH2 domain, which we have previously identified to be mandatory for Grb2-mediated Ca^{2+} inhibition. Interestingly, inducible tyrosine phosphorylation of Dok-3 requires the presence of the binding partner Grb2. This demonstrates the existence of a regulatory circuit, in which Grb2 'instructs' the kinase Lyn to create a specific docking site for the adaptor's SH2 domain. Second, *grb2*$^{-/-}$ and *dok-3*$^{-/-}$ cells exhibit almost identical Ca^{2+} profiles, which are characterized by robust intra- and extracellular Ca^{2+} fluxes. Hence, gene targeting of either *grb2* or *dok-3* is sufficient to convert the weak and monophasic Ca^{2+} response of an immature B cell such as DT40 to that of more mature B cells. Third, the dominant-negative $W^{193}K$ mutant of Grb2 cannot augment the Ca^{2+} response in *dok-3*$^{-/-}$ cells, demonstrating that Grb2 needs Dok-3 for Ca^{2+} signaling. Fourth and vice versa, the $Y^{331}F$ mutant of Dok-3, which cannot associate with Grb2 becomes hardly phosphorylated and is incapable of inhibiting BCR-induced Ca^{2+} mobilization. Moreover, all other tyrosine phosphorylation motifs of Dok-3, which bind other signaling proteins, are dispensable for Ca^{2+} inhibition. Fifth, membrane-associated Dok-3 is able to recruit and relocalize the majority of cytosolic Grb2 to the plasma membrane upon BCR activation. Loss of membrane association abrogates Dok-3 phosphorylation, association to Grb2 and concomitant Ca^{2+} inhibition. In summary, our data show that SH2 domain-mediated recruitment of Grb2 to Dok-3, which is tethered at the plasma membrane via its PH domain and becomes phosphorylated by Lyn, limits the quantity and quality of the BCR-induced Ca^{2+} signal.

Further studies presented in this paper excluded possible effector proteins of the Dok-3/Grb2 module. Importantly, we found no evidence for a role of SHIP, which was a likely candidate, because it is a reported Dok-3-binding protein (Lemay *et al*, 2000; Robson *et al*, 2004), and its lipid phosphatase activity reduces the number of membrane anchor motifs for PH domain-containing signaling proteins of the Ca^{2+}-activating pathway (Damen *et al*, 1996). We confirmed the *in vivo* association between SHIP and the PTB domain of Dok-3, but the Dok-3/Grb2 module neither requires this interaction nor the expression of SHIP at all to inhibit Ca^{2+} fluxes. Also the phosphorylation-dependent association of Dok-3 with Csk is dispensable for Ca^{2+} signaling and inhibitory phosphorylation of Lyn. Finally, we demonstrated homo-oligomerization of Dok-3 proteins mediated by the PTB domain and phospho-Y^{140}, but our mutational analysis of these intramolecular interaction sites excluded that this process participates in Ca^{2+} inhibition. Hetero-oligomerization between Dok-1 and Dok-2 had been previously reported to play a role in CD2 signaling in T cells (Boulay *et al*, 2005).

Two key observations provide mechanistic insight into the pathway downstream of the Dok-3/Grb2 module; that is, the substantially enhanced phosphorylation of PLC-γ2 at Y^{759} in *dok-3*$^{-/-}$ cells, which was accompanied by increased IP3 production and, the critical importance of the C-terminal SH3 domain of Grb2 for Ca^{2+} inhibition (see also Stork *et al*, 2004). The first results unmask Btk as the target of the Dok-3/Grb2 module because phosphorylation of Y^{759} in PLC-γ2 is strictly Btk-dependent and required for sustained Ca^{2+} elevation (Humphries *et al*, 2004; Kim *et al*, 2004). Our latter finding suggests how Dok-3-associated Grb2 can inhibit Btk function. Two conserved SH3 domain recognition motifs in the Tec homology (TH) domain of Btk are implicated in the regulation of kinase activity (Vihinen *et al*, 1996; Yamadori *et al*, 1999; Hansson *et al*, 2001; Okoh and Vihinen, 2002). Recruitment of Grb2 to phospho-Dok-3 may bring Grb2 into the vicinity of membrane-anchored Btk. Note that both Dok-3 and Btk are tethered at the plasma membrane through their PH domains and hence may reside in the same membrane sub-compartment. Colocalization of Dok-3/Grb2 with Btk could permit the C-terminal SH3 domain of Grb2 to bind the proline-rich regions in Btk and directly suppress its enzymatic activity. Alternatively, association between Grb2 and Btk occurs already in the cytosol, and phosphorylated Dok-3 targets the Grb2/Btk complex to a membrane compartment, where Btk cannot interact with PLC-γ2 for activation. For this purpose, PLC-γ2 needs to be located in lipid rafts, which explains why Grb2 recruitment to phosphorylated lipid raft residents, such as NTAL, facilitates sustained Ca^{2+} elevation (Stork *et al*, 2004). A sequestering function of the Dok-3/Grb2 module for Btk is supported by the ability of Dok-3 to inhibit Ras activation (Honma *et al*, 2006). In any

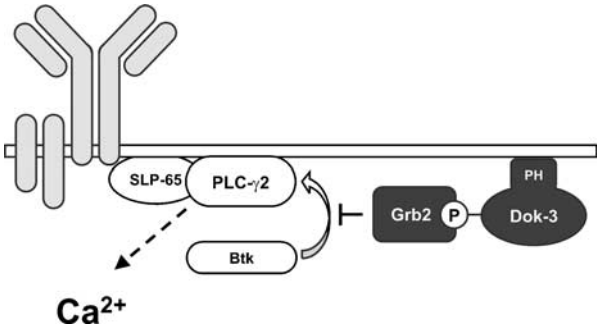

Figure 7 Inhibition of BCR-induced Ca^{2+} signaling by the Dok-3/Grb2 module. The Dok-3 adaptor protein is tethered at the inner side of the plasma membrane by virtue of its PH domain. Following tyrosine phosphorylation in activated B cells, Dok-3 recruits Grb2, which at this specific subcellular location attenuates Btk-dependent activation of PLC-γ2 by interfering with the proper formation of the SLP-65-assembled Ca^{2+} initiation complex and/or inhibiting the enzymatic activity of Btk. Positive and negative regulators of Ca^{2+} elevation are illustrated by open and filled boxes, respectively. The BCR complex is depicted in gray.

case phosphorylated Dok-3 appears to provide a 'membrane zip code' for Grb2 and the two models are not necessarily mutually exclusive (see Figure 7). A combination of biochemical and imaging methods will be required to finally elucidate the mode of action of the Dok-3/Grb2/Btk signaling module. It is tempting to speculate that the negative regulatory signal circuit described in this manuscript is involved in anergizing immature B cells upon auto-antigen encounter.

Materials and methods

Cells, Abs and reagents

Chicken DT40 cells were cultured in RPMI 1640 supplemented with 10% FCS, 1% chicken serum, 3 mM L-glutamine, 50 μM β-ME and antibiotics. Grb2- and SHIP-deficient DT40 cells are described by Ono *et al* (1997) and Hashimoto *et al* (1998). Mouse Bal17.TR B cells were kindly provided by Anthony de Franco (University of California, San Francisco, USA) and cultured in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 2 mM pyruvate, 50 μM β-ME and antibiotics (Harmer and DeFranco, 1999). Cell stimulation and lysis were performed as described by Stork *et al* (2004). Rabbit anti-mouse Dok-3 antibodies were kindly provided by André Veillette (IRCM, Montreal, Canada) (Lemay *et al*, 2000). Monoclonal antibodies to pTyr (4G10) and Grb2 (3F2) were purchased from Upstate Biotechnology (USA). Anti-Akt and phospho-specific antibodies to SHIP (Y^{1020}), Src (Y^{416}), Lyn (Y^{507}) and Akt (S^{473}) were purchased from Cell Signaling Technology (USA). Rabbit anti-PLC-γ2 (Q-20) and mAb rat anti-HA (3F10) were purchased from Santa Cruz Biotechnology (USA) and Roche (Switzerland), respectively. GST fusion proteins of Grb2[SH2] and Grb2[SH3] were prepared and used as described previously (Grabbe and Wienands, 2006).

Mass spectrometric analysis

DT40 (5×10^8) cells were stimulated with M4 and lysates were subjected to Grb2[SH2] affinity purification. After SDS–PAGE, proteins were visualized by silver staining. Proteins of interest were excised and in-gel-digested in an adapted manner according to Shevchenko *et al* (1996). Peptides generated were subjected to a 75-μm ID, 5-cm PepMap C18-column (Dionex, Germany). Peptide separation was performed by an acetonitrile gradient at 300 nl/min using an Ultimate/Swichos Nano-HPLC (Dionex, Germany) online coupled via a nano-spray source (Bruker, Germany) to a Esquire HCT Iontrap mass spectrometer (Bruker, Germany). Mass spectra were acquired in negative MS/MS mode, tuned for tryptic peptides. Processing of the MS/MS-spectra was performed by the use of Data Analysis and BioTools softwares from Bruker, Germany. Database search was done on the current NCBI protein database using an in-house MASCOT server.

Expression constructs and generation of Dok-3-deficient DT40 cells

The targeting vectors pDok-3-neo and pDok-3-hisD (see Supplementary Figure S2A) were constructed to insert neomycin and histidinol resistance cassettes into intron 1 of avian *dok-3* alleles. The resistance cassettes were flanked by 1.8 and 2.9 kb at the 5′- and 3′-sites, respectively. For this purpose, genomic *dok-3* fragments were amplified from DT40 genomic DNA using the primers 5′-TAGCACAGCTGTAGAGATGGCAGTG-3′ and 5′-AGCACATGAAGT CATCGCTTCTCC-3′ (left arm), and 5′-GCACGTTATGGGTGACAT CATGGCAG-3′ and 5′-GAAGATGTTCTCATAGAGATGCTCGG-3′ (right arm). Targeting vectors were introduced by electroporation at 550 V and 25 μF. For selection, G418 was used at 2 mg/ml and histidinol at 1 mg/ml. Dok-3-deficient clones were screened by PCR and immunoblot analysis. Further details of the targeting strategy and selection of Dok-3-deficient clones are described in Supplementary Figure S2. Full-length avian *dok-3* cDNA was obtained by RT–PCR with RNA from DT40 cells by using 5′-CAGTTGCTTTGGCTGAAT CAGTCAC-3′ and 5′-TTTTGTTACGGCGCCCCCTGGCGG-3′ as forward and reverse primers, respectively. The GenBank accession number of avian *dok-3* cDNA is EF051736. Wild-type chicken *dok-3* cDNA was cloned into pCRII-TOPO vector. Coding sequences for HA tag were introduced at the 5′- and eGFP at the 3′-sites of the Dok-3 cDNA by PCR. The different Dok-3 variants were obtained using site-directed mutagenesis. All *dok-3* cDNAs were directly ligated into the expression vectors pMSCV (BD Biosciences, USA) and pApuroII (Kurosaki *et al*, 1994). The generation of Grb2 constructs (GenBank accession number EF062570) and retroviral transduction or electroporation of expression vectors are described in Stork *et al*, (2004).

Calcium and IP3 measurements

For Ca^{2+} monitoring, 1×10^6 cells were loaded in 700 μl RPMI containing 5% FCS, 1 μM Indo1-AM (Molecular Probes) and 0.015% Pluronic F127 (Molecular Probes, USA) at 30°C for 25 min. Subsequently, the cell suspension was diluted two-fold with RPMI 10% FCS and incubated for 10 min at 37°C. Cells were washed and prepared for measurements as described earlier (Stork *et al*, 2004). Briefly, to discriminate between mobilization of Ca^{2+} from intra- and extracellular sources, BCR stimulation was performed for 6 min in the presence of 0.5 mM EGTA to remove extracellular Ca^{2+} and to allow for monitoring Ca^{2+} release from ER stores only. After restoring the extracellular Ca^{2+} concentration to 1 mM, entry of Ca^{2+} through ion channels in the plasma membrane was recorded. Changes in the ratio of fluorescence intensities at 405 and 510 nm were monitored on an LSRII cytometer (Becton Dickinson) and analyzed with FlowJo (TriStar). IP3 concentrations were determined using the IP3 Biotrak Assay (GE Healthcare) according to the manufacturer's protocol.

Confocal laser scanning microscopy

A total of 1×10^6 DT40 cells were resuspended in Krebs Ringer solution composed of 10 mM HEPES (pH 7.0), 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose. After 30 min of seeding onto chambered coverglasses (Nunc, USA), cells were examined on a Leica TCS SP2 confocal laser scanning microscope. EGFP was excited at a wavelength of 488 nm and emission was recorded at 510 nm.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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