On adaptor proteins shaping Ca^{2+} signals in activated B lymphocytes

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Contents

I Abbreviations

dH2O	deionized water
$[Ca^{2+}]_i$	intracellular Ca^{2+} concentration
APS	ammonium persulfate
BCAP	B cell adaptor for PI3K
BCR	B cell antigen receptor
BLNK	B cell linker protein
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
Ca^{2+}	Calcium ion
CRAC	Ca^{2+} release activated Ca^{2+} channel
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
dNTP	2'-deoxynucleoside-5'-trisphosphate
Dok	downstream of kinase
DTT	1,4-dithiothreitol
ECL	enhanced chemical luminescence
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene-glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
$F(ab')_2$	bivalent antigen-binding fragment
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
GEF	guanine-nucleotide exchange factor
Grb2	growth factor receptor-bound protein 2
HPK1	hematopoietic progenitor kinase 1
HRPO	horseradish peroxidase
Ig	immunoglobulin

Indo-1	1H-indole-6-carboxylic acid, 2-[4-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-3-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-, (acetyloxy)methyl ester
IP	immunoprecipitation
IP3	inositol-1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
LAT	linker for activation of T cells
LB	lysogeny broth
Lyn	lck/yes-related novel PTK
NCBI	National Center for Biotechnology information
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor for κ gene in B lymphocytes
NTAL	non-T cell activation linker
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PH	pleckstrin homology
$PI(3,4)P_2$	phosphatidylinositol 3,4-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-trisphosphate
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol 3'-kinase
PLC-γ1/2	phospholipase C-γ1/2
PTB	phosphotyrosine binding
PTK	protein tyrosine kinase
pTyr	phosphotyrosine
Ras	abbreviation originated from rat sarcoma
RasGRP	Ras guanine nucleotide releasing protein
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RTK	receptor tyrosine kinase
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase

SERCA	sarcoplasmic endoplasmic Ca^{2+} ATPase
SH	Src homology
Shc	SH2-containing sequence
SHIP	SH2 domain-containing inositol 5'-phosphatase
SLP-65	SH2 domain-containing leukocyte adaptor protein of 65 kDa
SOCE	store-operated Ca^{2+} entry
SOS	son of sevenles
src	Rous sarcoma oncogene
STIM	stromal interaction molecule
Syk	Spleen tyrosine kinase
TBS	Tris-buffered saline
Tris	Tris-(hydroxymethyl)-aminomethane
VSV-G	vesicular stomatitis virus glycoprotein

II Amino Acids

Amino Acid	3-letter code	symbol
Alanine	Ala	A
Cysteine	Cys	C
Aspartic Acid	Asp	D
Glutamic Acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

III Deoxyribonucleotides

Deoxyribonucleotides	symbols
deoxyadenosine monophosphate	a, A
deoxycytidine monophosphate	c, C
deoxyguanosine monophospate	g, G
deoxythymidine monophosphate	t, T

IV Prefixes & Units

M	mega	m	meter	Bq	becquerel
k	kilo	L	liter	Ci	Curie
c	centi	M	molar	V	volt
m	milli	g	gram	A	ampere
µ	micro	b	base	F	farad
n	nano	bp	base-pair	h	hour
p	pico	Da	Dalton	min	minute
U	unit	s	second	*g*	acceleration of gravity
°C	degree Celsius	rpm	rounds per minute		

1 Summary

Signals emanating from the B cell antigen receptor (BCR) regulate both the activation of pathogen-specific B lymphocytes as well as removal of self-reactive B lymphocytes. The outcome of BCR stimulation can range from induction of apoptosis to proliferation. Inappropriate signals can therefore lead to an imbalance between humoral immunity and self tolerance. Consequently, B lymphocyte activation depends on a complex interplay between positive and negative signaling elements. The rise of the cytosolic Ca^{2+} concentration is a central step in B lymphocyte activation. The temporal shape of the Ca^{2+} signal contributes to the differential activation of transcription factors, which then affects cell fate decisions.

The adaptor protein Grb2 has previously been shown to inhibit BCR-induced Ca^{2+} mobilization. During my work Björn Stork in our lab identified Dok-3 to be another adaptor protein inhibiting Ca^{2+} mobilization in B cells. In this study I investigated the molecular mechanisms of the inhibitory function of the two adaptor proteins.

Using DT40 B cells that were rendered deficient for Grb2 or Dok-3 expression it could be shown that both proteins inhibit activation of the Ca^{2+} mobilizing enzyme PLC-γ2 by Btk. This was evident from enhanced BCR-induced phosphorylation of PLC-γ2 at a specific Btk phosphorylation site in the absence of Dok-3 or Grb2. This finding was supported by enhanced IP3 production and a stronger degree of depletion of Ca^{2+} from the ER stores in response to BCR ligation. An impact of the two adaptor proteins on the activation of plasma membrane Ca^{2+} channels could be excluded by uncoupling the release of Ca^{2+} from the ER from Ca^{2+} influx across the membrane. Although both proteins seem to form a ternary complex with the inhibitory lipid phosphatase SHIP, this interaction could be shown not to be essential for Dok-3 function. Rather, SHIP seems to promote Dok-3 phosphorylation. FcγRIIb coligation to the BCR strongly activates SHIP and this could be shown to also enhance Dok-3 phosphorylation.

Together, this study reveals insights in the molecular mechanism by which the cytosolic adaptor proteins Dok-3 and Grb2 regulate BCR signaling and identifies FcγRIIb as an upstream regulator.

1.1 Zusammenfassung

Signale des B Zell Antigenrezeptors (BZR) regeln sowohl die Aktivierung pathogenspezifischer B Lymphozyten als auch die Eliminierung selbstreaktiver B Lymphozyten. Das Ergebnis der BZA Stimulation kann von Apoptose bis zu Proliferation der B Lymphozyten reichen. Unpassende Signale können deshalb zu einer Unausgewogenheit zwischen der humoralen Immunität und Selbsttoleranz führen. Folglich hängt die B Lymphozyten-Aktivierung von einem komplizierten Wechselspiel zwischen positiven und negativen Signalelementen ab. Der Anstieg der zytosolischen Kalziumionen (Ca^{2+}) Konzentration ist ein zentraler Schritt in der B Lymphozyten-Aktivierung. Die zeitliche Form des Ca^{2+} Signals trägt zur differentiellen Aktivierung von Transkriptionsfaktoren bei, welche dann das Schicksal der Zelle beeinflussen. Das Adapterprotein Grb2 hemmt BZR-vermittelte Ca^{2+}-Mobilisierung. Während meiner Arbeit identifizierte Björn Stork in unserem Labor Dok-3 als ein weiteres Adapterprotein, das Ca^{2+} Mobilisierung in B Zellen hemmt.

In dieser Arbeit habe ich die molekularen Mechanismen der hemmenden Funktion der zwei Adapterproteine. untersucht. Dafür wurden DT40 B-Zellen verwendet, deren Gene für Grb2 oder Dok-3 inaktiviert worden sind. Damit konnte gezeigt werden, dass beide Proteine die Aktivierung des Ca^{2+}-mobilisierenden Enzyms PLC-γ2 durch Btk hemmen. Dies zeigte sich durch eine erhöhte BZA-vermittelte Phosphorylierung von PLC-γ2 an einer spezifischen Btk-Phosphorylierungsstelle in der Abwesenheit von Dok-3 oder Grb2. Dieser Befund wurde durch erhöhte IP3-Produktion und einen stärkeren Grad der Entleerung der intrazellulären Ca^{2+}-Speicher nach BZR-Stimulation unterstützt. Der beobachtete Einfluss der zwei Adapterproteine auf der Aktivierung von Ca^{2+}-Kanälen in der Plasmamembran verschwindet, wenn die Speicher künstlich entleert sind. Daher scheinen die beiden Adapterproteine ausschließlich die Ca^{2+} Mobilisierung aus intrazellulären Speichern zu beeinflussen. Obwohl beide Proteine einen ternären Komplex mit dem hemmenden Lipidphosphatase SHIP bilden, scheint diese Wechselwirkung für die Dok-3-Funktion nicht notwendig zu sein. Eher scheint SHIP die Dok-3-Phosphorylierung zu fördern. Koquervernetzung von FcγRIIb mit dem BZR führt zu starker SHIP-Aktivierung und ich konnte zeigen, dass dies auch zu verstärkter Dok-3-Phosphorylierung führt. Diese Studie zeigt Einblicke in die molekularen Mechanismen, durch die die zytosolischen Adapterproteine Dok-3 und

Grb2 BZR-Signale regeln und identifiziert FcγRIIb als Regulator des Dok-3-Grb2-Moduls.

2 Introduction

2.1 The Immune System

The vertebrate immune system is comprised of a diverse set of leukocytes emanating from pluripotent hematopoietic stem cells that secure the survival of the organism by detecting and eliminating pathogens.

The immune system can be divided into two interconnected subsystems: The innate immune system recognizes and attacks pathogens based on common pathogen-associated molecular patterns (PAMPs), like bacterial cell wall constituents. Recognition of the pathogen by components of the innate immune system also leads to the activation of the adaptive immune system, which produces cells that specifically recognize the invading pathogen. Adaptive immunity can be divided into humoral and cellular immunity. Humoral immunity is mediated by antibodies and is directed against extracellular pathogens. Antibodies are secreted proteins that specifically bind antigens of the pathogen and binding leads to neutralization or targeting of the pathogen for elimination by various effector mechanisms. Cellular immunity is mediated by T lymphocytes and is directed against intracellular pathogens, which are inaccessible to antibodies. T lymphocytes destroy intracellular pathogens by either activating macrophages to kill the pathogen or by killing infected cells to eliminate reservoirs of infection.

The humoral immune response is based on a large repertoire of randomly rearranged antigen receptor genes. These genes are expressed in lymphocytes, each lymphocyte carrying only one rearranged gene and thus only one antigen receptor. Binding of these receptors to their cognate antigen leads to the production of large amounts of the receptor in soluble form, which are then called antibodies. This system, which has essentially been postulated by Paul Ehrlich in his “side chain theory” (Ehrlich, 1900), has evolved in parallel in jawless fish using modules of leucine-rich repeats (Alder et al., 2008; Pancer et al., 2004) and in higher vertebrates using immunoglobulin gene segments (Cooper and Alder, 2006).

2.2 Antibody production by B lymphocytes in higher vertebrates

The antigen receptors of the higher vertebrate humoral immune system are expressed as membrane-bound immunoglobulins at the surface of B lymphocytes and together with two signaling subunits, Igα and Igβ, comprise the B cell antigen receptor (BCR). Binding of antigen to its cognate BCR leads to internalization of the receptor and transport of antigen to endolysosomes, where the antigen is processed and loaded on MHC class II molecules. T helper cells, recognizing the antigen loaded MHC II, then activate the B cell and induce the germinal center reaction.

Unlike T cells, B cells respond to unprocessed extracellular antigens. Thus, they face the problem that these antigens are extremely diverse; they may be soluble or particulate, contain a single or multiple epitopes, may or may not be opsonizable by molecules of the innate immune system (complement, pattern recognition molecules or natural antibodies) and may be available in very low to very high concentrations. To challenge these problems, the immune system seems to have evolved many different ways to activate B cell subsets. A recent report shows that soluble antigen can passively diffuse into B cell follicles of lymph nodes where it encounters B cells (Pape et al., 2007). Another report for the first time shows, that B cells can be activated by dendritic cells (DCs) presenting native antigen on their surface (Qi et al., 2006). It has been noted previously that B cells are activated much more efficiently by antigen displayed on cellular surfaces. Groundbreaking work in the laboratory of Facundo Batista showed that B cells sense the affinity to membrane-bound antigens by spreading on the displaying surface forming a immunological synapses similar to T cells (Batista et al., 2001; Fleire et al., 2006). Supporting the importance of membrane-bound native antigens, a very recent report shows that macrophages of the lymph node subcapsular sinus facilitate activation of B cells by collecting particulate antigen from the lymph and transporting it in native form into the B cell follicle (Carrasco and Batista, 2007; Junt et al., 2007). In a similar manner marginal zone B cells appear to transport opsonized antigen from the marginal zones of the spleen to the follicles (Cinamon et al., 2008). These studies indicate how B cells could also efficiently respond to antigen with only one epitope or how antigen can be concentrated for B cell activation.

Besides activation of mature B cells, the signal emanating from the BCR plays a pivotal role in the development of B cells. Signaling by the pre-BCR is required for the transition of pro B cells to the pre B cell stage and signaling by the nascent BCR is required for transition of pre B cells to the immature B cell stage. Further, BCR signals are required for induction of tolerance by deletion of autoreactive B cells, B cell selection in the germinal centers and reactivation of memory B cells. (King and Monroe, 2000; Wang and Clark, 2003))

2.3 Regulation of cytosolic Ca^{2+} concentration in B cells

Ca^{2+} ions act as second messenger downstream of various receptors. The intracellular Ca^{2+} concentration in eukaryotic cells has to be kept very low, since many phosphorylated compounds form insoluble precipitates with Ca^{2+}. This and other facts make the Ca^{2+} ion an ideal second messenger in intracellular signal transduction. Ca^{2+} can bind to various proteins and induce a conformational change upon a transient rise in cytosolic Ca^{2+} concentration (Williams, 1998).

In B cells, the rise in cytosolic Ca^{2+} concentration in response to BCR ligation by antigen is a central step in the activation of several signal-transduction pathways, leading to activation of key transcriptions factors like nuclear factor of activated T cells (NFAT) and NF-κB (Engelke et al., 2007).

BCR ligation triggers elevation of intracellular Ca^{2+} concentrations through activation of phospholipase C-γ2 (PLC-γ2), which in turn hydrolyzes membrane phospholipids to yield soluble inositol 1,4,5-trisphosphate (IP3). IP3 receptors are ligand gated Ca^{2+} channels located in the membrane of the endoplasmic reticulum (ER), which is the main storage for intracellular Ca^{2+}. Hence, IP3 production causes release of Ca^{2+} from the ER to the cytosol, which results in a first rise in intracellular Ca^{2+} concentration. Since the amount of Ca^{2+} in the ER is limited, this Ca^{2+} release is transient. The IP3-driven Ca^{2+} release is followed by entry of Ca^{2+} ions from the extracellular space through calcium-release-activated calcium (CRAC) channels, which contain ORAI-family proteins (Feske et al., 2006; Yeromin et al., 2006). This process is induced by depletion of the ER Ca^{2+} stores and is thus referred to as store-operated Ca^{2+} entry (SOCE). The reduced Ca^{2+} concentration of the ER is thought to induce a conformational change in stromal

interaction molecule (STIM) proteins, which are transmembrane proteins of the ER membrane. This leads to STIM binding to CRAC channels and activation of the latter (Wu et al., 2006; Zhang et al., 2005). The phase of Ca^{2+} entry across the plasma membrane can last longer than the first, since the amount of Ca^{2+} in the extracellular space is not as limited as the intracellular stores. Importantly, SOCE is only activated, when a certain threshold of IP3 accumulation and store depletion is reached (Parekh et al., 1997). This has the effect of allowing small differences in the level or duration of PLC activation to lead to very large differences in the shape and especially in the duration of the Ca^{2+} signal. The shape of the Ca^{2+} signal leads to activation of distinct transcription factors and is thought to contribute to cell fate determination like proliferation, anergy or apoptosis (Dolmetsch et al., 1997).

Independent of the amount of Ca^{2+} released from the ER, the cytosolic Ca^{2+} concentration can be regulated by several other mechanisms. The driving force of Ca^{2+} across the plasma membrane is strongly dependent on the plasma membrane potential. BCR ligation has indeed been shown to induce membrane depolarization in a B cell line (Hashimoto et al., 2001). Further, Ca^{2+} can be actively transported out of the cytosol into the extracellular space or back into the ER by plasma membrane Ca^{2+} ATPases (PMCAs) or sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCAs), respectively. The negative co-receptor CD22 has been shown to activate PMCAs to limit rise in intracellular Ca^{2+} concentration (Chen et al., 2004). Furthermore, mitochondria have been shown to buffer cytosolic Ca^{2+} by passive uptake of Ca^{2+} and extrusion through unknown Na^{+} or H^{+}- gradient-driven active transporters (Chen et al., 2004; Jacobson and Duchen, 2004).

2.4 Activation of PLC-γ2 downstream of the BCR

Activation of PLC-γ2 requires recruitment of PLC-γ2 to the inner leaflet of the plasma membrane, phosphorylation by Bruton´s tyrosine kinase (Btk) and availability of its substrate phosphatidyl-inositol-4,5-phosphate ($PI(4,5)P_2$). BCR crosslinking induces activation of tyrosine kinases of the Src and Syk family, which leads to phosphorylation and membrane recruitment of the central adaptor protein SLP-65 (Chiu et al., 2002; Fu et al., 1998; Wienands et al., 1998). Phosphorylated SLP-65 then recruits PLC-γ2 and Btk to the plasma membrane and into a ternary complex, called Ca^{2+} initiation complex

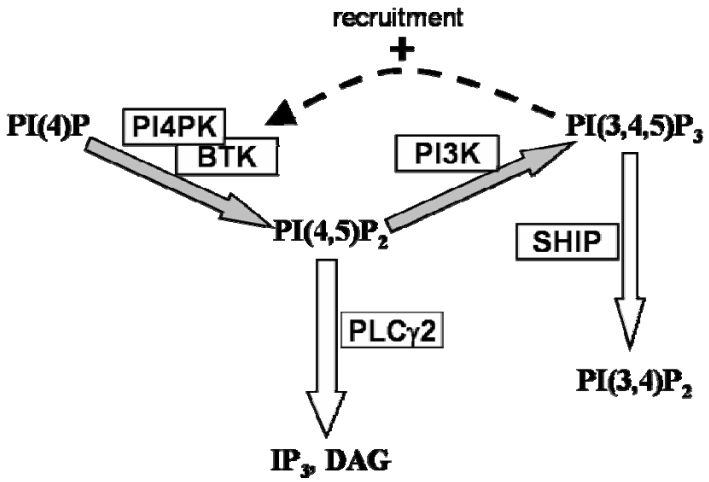

Figure 1: Positive feedback loop in BCR signaling. See text for details

(Chiu et al., 2002; Su et al., 1999). This enables phosphorylation of PLC-γ2 by Btk, which is essential for activation of its catalytic activity.

In parallel the activation of phosphoinositide 3-kinase (PI3K) by pathways involving CD19 or B cell adaptor for PI3K (BCAP) leads to the production of phosphatidyl-inositol-3,4,5-phosphate ($PI(3,4,5)P_3$) from $PI(4,5)P_2$, which is also the substrate for PLC-γ2 (Aiba et al., 2008). $PI(3,4,5)P_3$ binds with high affinity to the pleckstrin-homology (PH) domain of Btk, enhancing its recruitment to the membrane (Bolland et al., 1998). As Btk constitutively interacts with phosphatidylinositol-4-phosphate 5-kinase (PIP5K), recruitment of Btk also brings PIP5K to the membrane and into the same complex (Carpenter, 2004; Saito et al., 2003). PIP5K promotes the conversion of PI(4)P to PI(4,5)P2, replenishing the substrate of PI3K and PLC-γ2. In this way, PI3K and Btk are part of a forward feedback loop (see Figure 1).

2.5 Regulation of PLC-γ2 activation by positive and negative co-receptors

The exceptional role of the above forward feedback loop initiated by PI3K on the outcome of BCR signaling is shown by similar B cell phenotype in mice deficient for Btk, SLP-65, PLC-γ2 and PI3K (Scharenberg et al., 2007). Further, this forward feedback loop is target of several signal modulating co-receptors.

CD19 is one of the best studied co-receptors of the BCR and is part of a complex comprising also TAPA-1 and complement receptor 1 (CR1). Binding of the BCR to antigen, which is opsonized by complement, leads to co-ligation of the BCR with this complex. This brings CD19 close to the activated Src family kinases at the BCR, and leads to its phosphorylation and recruitment of the regulatory subunit of PI3K, namely p85. PI3K is in turn activated leading to the induction of the above mentioned feedback loop. Co-ligation of CD19 with the BCR thus lowers the threshold of antigen required to induce BCR signaling (Fearon and Carroll, 2000).

Conversely, one of the best studied inhibitory co-receptors acts in a directly opposite way. Fcγ receptor IIb (FcγRIIb) is a low affinity receptor for the Fc part of IgG. It binds to immune complexes, a multivalent antigen opsonized with antibodies. When co-ligated to the BCR, it inhibits the PI3K pathway. Recruitment of FcγRIIb to the BCR enables phosphorylation of its cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM). This in turn recruits mainly the SH2 domain-containing inositol 5-phosphatase (SHIP), which hydrolyses the product of PI3K, namely $PI(3,4,5)P_3$, at the 5´position to $PI(3,4)P_2$. Note that $PI(3,4)P_2$ is distinct from the PI3K and PLCγ2 substrate $PI(4,5)P_2$. Degradation of $PI(3,4,5)P_3$ blocks PH domain-mediated Btk recruitment to the membrane and thus replenishment of $PI(4,5)P_2$ from Btk-associated PIP5K (see Figure 1). Thus, PLC-γ2 signaling is drastically reduced due to limitation of its substrate, and block of Btk recruitment by $PI(3,4,5)P_3$ (Nimmerjahn and Ravetch, 2006).

FcγRIIb has been shown to be essential for establishing peripheral tolerance (Tarasenko et al., 2007). Mice rendered deficient for FcγRIIb expression spontaneously develop a disease similar to systemic lupus erythematosus (SLE), which is characterized by the production of self antigen recognizing antibodies (Bolland and Ravetch, 2000; Takai et al., 1996). Since the autoimmune phenotype is strain dependent, other epistatic modifiers seem to be involved in disease susceptibility and severity (Bolland et al., 2002). Also in humans mutations in the gene encoding FcγRIIb have been linked to susceptibility to SLE. A polymorphism in the human FcγRIIb promoter that reduces its expression and a mutation in the transmembrane domain of the receptor that blocks its

signaling capability have been described (Blank et al., 2005; Chen et al., 2006; Chu et al., 2004; Kono et al., 2005)

2.6 Role of Dok-3 and Grb2 in BCR signaling

Adaptor proteins are devoid of catalytic activity and signal by assembling signaling complexes, relocating enzymatic entities to subcellular locations or altering enzyme activities (Pawson, 2007; Wienands, 2000). Previously, two adaptor proteins have been shown to inhibit Ca^{2+} mobilization in response to BCR stimulation, namely Grb2 (growth factor receptor-bound protein 2) and Dok-3 (downstream of kinase-3). Grb2 is composed of a central SH2 domain flanked on either side by one SH3 domain (Figure 2) (Lowenstein et al., 1992). It is expressed in all cell types and throughout the B cell lineage. B cells rendered deficient for Grb2 showed enhanced Ca^{2+} mobilization in response to BCR stimulation, depending on the central SH2 and the C-terminal SH3 domain (Stork et al., 2004). The role of Grb2 in signaling downstream of receptor tyrosine kinases (RTKs) is well established. Both SH3 domains were demonstrated to bind the guanine nucleotide exchange factor (GEF) son of sevenless (SOS) (Buday and Downward, 1993). Binding of the central SH2 domain of Grb2 to activated receptors can thus recruit SOS to the membrane to activate the Ras/MAPK pathway (Egan et al., 1993). Downstream of the BCR, activation of the Ras/MAPK pathway is independent of Grb2, though. In this scenario, Ras is activated by RasGRPs (Ras guanine nucleotide release protein) (Oh-hora et al., 2003). The mechanism of the negative regulatory role of Grb2 on Ca^{2+} signaling remains elusive, but during this thesis Björn Stork found that Grb2 associates via its SH2 domain with Dok-3 after BCR stimulation. As Dok-3 is also

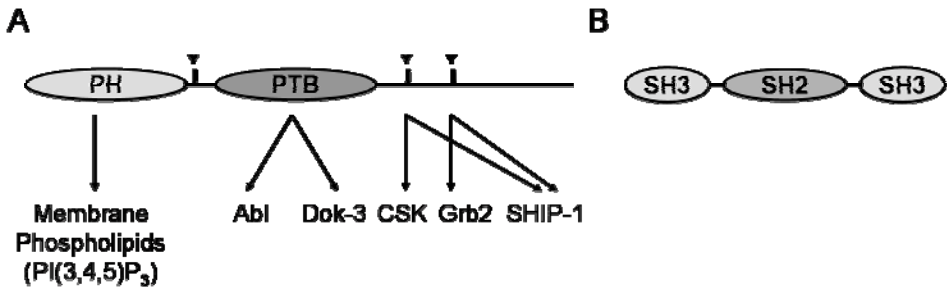

Figure 2 (A) Structural elements and known binding partners of Dok-3. Adapted from (Campbell, 2007)
(B) Domain structure of Grb2

an adaptor protein, Grb2 and Dok-3 probably associate with another protein that is involved in signal transduction. Grb2 has been shown to interact *in vivo* or *in vitro* with roughly 190 proteins (Human Protein Reference Database). The mode of interaction, direct vs. indirect and the domains involved are unclear for most of them. Of the described interaction partners many have been described to be involved in BCR signaling, e.g. Dok-3, Vav, SLP-65, p85, HPK1, Cbl, CIN85, SHC, NTAL/LAT, CD19 and SHIP (Brooks et al., 2000; Grabbe and Wienands, 2006; Harmer and DeFranco, 1999; Honma et al., 2006; Huang et al., 2000; Johmura et al., 2003; Kavanaugh et al., 1996; Kirsch et al., 1999; Oehrl et al., 1998; Stork, 2006; Weinger et al., 2008; Wienands et al., 1998; Ye and Baltimore, 1994)

The family of Dok proteins comprises 7 members, which are cytosolic adaptor proteins. Dok-1 (alternatively called p62Dok), Dok-2 (alternatively called DokR) and Dok-3 (alternatively called DokL) are predominantly expressed in cells of the hematopoietic lineage. They have a similar domain structure of an N-terminal PH domain followed by a phosphotyrosine-binding (PTB) domain and several tyrosine-based signaling motifs (see Figure 2 A). Dok-3 is expressed in B cells and myeloid cells. Upon BCR stimulation, it is tyrosine-phosphorylated and associates with SHIP, Grb2 and CSK (Lemay et al., 2000; Stork, 2006). Studies using overexpression of wild-type or a dominant negative mutant of Dok-3 in a B cell line suggested a selective role for the inhibition of NFAT and JNK activation downstream of the BCR, which was dependent on interaction with SH2 domain-containing 5`-inositol phosphatase (SHIP) (Robson et al., 2004). Ca^{2+} mobilization was not affected, though, which was surprising due to the well documented role of SHIP in Ca^{2+} inhibition. However, during this study, Björn Stork showed that DT40 B cells rendered deficient for Dok-3 show drastically enhanced Ca^{2+} mobilization in response to BCR stimulation. Grb2 seems to be involved in this inhibition as it is required for efficient phosphorylation of Dok-3. Together, the adaptor proteins Grb2 and Dok-3 seem to act as inhibitory signaling elements downstream of the BCR. Aim of this study was to elucidate the molecular mechanisms of the inhibition and to identify the catalytic entity regulated.

3 Aims of Work:

This thesis addressed the question of how the small adaptor protein Grb2 is involved in signaling downstream of the BCR. It has previously been shown that Grb2 inhibits BCR-induced Ca^{2+} mobilization, especially Ca^{2+} influx from the extracellular space (Stork, 2004). The molecular mechanism of this phenomenon was unknown. During my work, Björn Stork in our laboratory found Dok-3 to interact with Grb2 in response to BCR ligation. He could further show, that Dok-3-deficient B cells showed a similar Ca^{2+} phenotype and thus postulated Dok-3 and Grb2 to constitute one signaling module together.

DT40 B cells that were rendered deficient for Grb2 and Dok-3 were used to:

1. characterize the impact of Grb2 and Dok-3 on the two phases of BCR-induced Ca^{2+} mobilization.
2. identify the catalytic entity that is regulated by Grb2 and Dok-3.
3. compare the mode of action of Grb2 and Dok-3 to test the hypothesis that they constitute a signaling module together.

4 Materials and Methods

4.1 Materials

All chemicals and biologically reactive reagents were purchased from Roth, Sigma-Aldrich, Merck, Invitrogen (Gibco; Molecular Probes), Becton Dickinson, or GE Healthcare. All chemicals were purchased in pA quality unless otherwise indicated.

4.1.1 Antibodies

Table 1: Primary antibodies (WB, Western blot; IP, immunoprecipitation)

Antibody	supplier/reference	application
mouse anti-Grb2 (3F2)	Upstate	WB
rabbit anti-actin	Sigma	WB
rabbit anti-PLC-γ2 (Q-20)	Santa Cruz Biotechnolgy	WB, IP
mouse anti-phosphotyrosine (pTyr) (4G10)	Upstate	WB
rat anti-HA* (3F10)	Roche	WB, IP
rabbit anti-mouse Dok-3	(Lemay et al., 2000)	WB
goat anti-mouse Dok-3 (D-18)	Santa Cruz Biotechnology	IP
rabbit anti-chicken Dok-3	Lösing, 2007	WB, IP
mouse anti-chicken IgM (M4) hybridoma	(Chen et al., 1982) (Riken Cell Bank 1611)	DT40 stimulation
goat anti-chicken IgM FITC	Bethyl Laboratories	FACS analysis
rabbit anti-SHC	Becton Dickinson	WB, IP
goat anti-Dok-1	Santa Cruz Biotechnolgy	IP

rabbit anti-Dok-1	Santa Cruz Biotechnolgy	WB
mouse anti-SHIP (P1C1)	Santa Cruz Biotechnolgy	WB
rabbit anti-phospho-SHIP(Y1020)	Cell signaling technology	WB
rabbit anti-phospho-Akt(S473)	Cell signaling technology	WB
rabbit anti-Akt	Cell signaling technology	WB
rabbit anti-phospho-Lyn(Y507)	Cell signaling technology	WB
rabbit anti-phospho-SRC(Y416)	Cell signaling technology	WB
rabbit anti-phospho-PLC-γ2 (Y759)	Cell signaling technology	WB
rabbit anti-Btk	Santa Cruz Biotechnolgy	WB
rat anti-mouse CD16/CD32 (2.4G2)	Becton Dickinson	Fc receptor blocking
F(ab')2 fragment goat anti-mouse IgM, μ-chain specific	Jackson ImmunoResearch	mouse B cell stimulation
rabbit-anti-mouse IgM, μ-chain specific	Jackson ImmunoResearch	mouse B cell stimulation

4.1.2 Enzymes

Restriction endonucleases	New England Biolabs
Calf Intestine Phosphatase (CIP)	New England Biolabs
T4 DNA Ligase	New England Biolabs
Taq DNA polymerase	New England Biolabs
Pfu DNA polymerase	Promega
Moloney murine leukemia virus reverse transcriptase (MMLV-RT)	MBI Fermentas
Topoisomerase	Invitrogen

4.1.3 Vectors and cDNAs

Table 2: Vectors

Vector	supplier/source	application
pCR2.1 Topo	Invitrogen	T/A cloning
pApuroII	T. Kurosaki	High expression of proteins
pMSCVpuro	BD Biosciences Clontech	Expression of Dok-3, and Grb2
pLNCX2	BD Biosciences Clontech	Expression of SHIP cDNA
pHCMV-VSV-G	M. Jücker	expression of VSV-G/ pseudo-typing of retroviruses

Table 3: cDNAs

cDNA	supplier/source	expression vectors
chicken *dok-3*	B. Stork	pMSCVpuro
chicken *grb2*	B. Stork	pMSCVpuro
mouse *grb2*	A. Grabbe	pMSCVpuro
mouse *ship*	J. Frey	pLNCX2
chicken *btk*	cloned from DT40 mRNA	pMSCVpuro, pApuro
human *fcgr2b*	J. Frey	pBEHpac18

4.1.4 Oligonucleotides

All oligonucleotides were synthesized by MWG-Biotech (Ebersberg)

Table 4: Oligonucleotides

Primer	sequence	application
chicken Btk fw Xba	ctt cta gag ctg ggg tgg ccg aca	cloning of chicken Btk cDNA
chicken Btk rev HA XhoI	gtc aag cgt agt ctg gga cgt cgt atg ggt acg gct ctt cgt ctg tga tg	cloning of chicken Btk cDNA
mouse SHIP Y917F fw	atg atc aat cca aac ttc att ggt atg ggg c	mutagenesis of SHIP cDNA
mouse SHIP Y917F rev	gcc cca tac caa tga agt ttg gat tga tca	mutagenesis of SHIP cDNA
mouse SHIP Y1020F fw	gaa gga act cac gga tcc aaa cag tgg gtt ctc aaa	mutagenesis of SHIP cDNA
mouse SHIP Y1020F rev	ttt gag aac cca ctg ttt gga tcc gtg agt tcc ttc	mutagenesis of SHIP cDNA
mouse SHIP ΔIPase fw	caa cag gga tga agt acc tgg tgc atg tgg tct g	mutagenesis of SHIP cDNA
mouse SHIP ΔIPase rev	cag acc aca tgc acc agg tac ttc atc cct gtt g	mutagenesis of SHIP cDNA
mouse SHIP K675A fw	cgt cct ggt gcg ccc gtc ctg gtg gtg cgc cgc ccg agt cct ctg gaa g	mutagenesis of s SHIP hip cDNA
mouse SHIP K675 rev	ctt cca gag gac tcg ggc gca cca gga cgg gcg cac cag gac g	mutagenesis of SHIP cDNA
Y140-FSSW fwd	gga gaa ctg cct ctt ctc ctc gtg gca gg	mutagenesis of chicken Dok-3 cDNA
Y140-FSSW rev	cct gcc acg agg aga aga ggc agt tct cc	mutagenesis of chicken Dok-3 cDNA
R197A fwd	gcc cta ccc gtt cct cgc caa att cgg cca aga tc	mutagenesis of chicken Dok-3 cDNA
R197A rev	gat ctt ggc cga att tgg cga gga acg ggt agg gc	mutagenesis of chicken Dok-3 cDNA
Y307-FASI fwd	ctg ccc cta tag tgt ttg cct cca tcg cac g	mutagenesis of chicken Dok-3 cDNA
Y307-FASI rev	cgt gcg atg gag gca aac act ata ggg gca g	mutagenesis of chicken Dok-3 cDNA

Y331-FENI fwd	ctc ccc gag cat ctc ttt gag aac atc ttc acg	mutagenesis of chicken Dok-3 cDNA
Y331-FENI rev	cgt gaa gat gtt ctc aaa gag atg ctc ggg gag	mutagenesis of chicken Dok-3 cDNA

4.1.5 Solutions, buffers, and media

The compositions of all solutions and buffers used are listed in the sections in which the corresponding experimental procedures are described. All solutions and buffers are aqueous solutions and stored at room temperature unless otherwise indicated. If solutions, buffers, or media had to be autoclaved it is indicated. Autoclaving of liquids was carried out at 125 °C for 30 min. RPMI medium was purchased endotoxin free and filter sterilized (Invitrogen).

The following common buffers were used:

PBS ..... 137 mM NaCl; 27 mM KCl; 43 mM Na_2HPO_4 x 7 H_2O; 14 mM KH_2PO_4; pH ~ 7.3

TBS ..... 20 mM Tris/HCl pH 7,6; 137 mM NaCl

TE buffer ..... 10 mM Tris/HCl, pH 8.0; 1 mM EDTA, pH 8.0

4.1.6 Bacteria strains

Production of Plasmid DNA was achieved by transforming Top10F´ bacteria as described in the corresponding sections.

Genotype of Top10F':

F'[$lacI^q$ Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ-

4.1.7 Additional materials

GeneRuler™ 1 kb DNA ladder (MBI Fermentas)

Prestained Protein Marker, Broad Range (6.5-175 kDa) (New England Biolabs)

Protease Inhibitor Cocktail (P 2714) (Sigma-Aldrich)

Tissue culture equipment (dishes, pipettes, tubes etc.) (Greiner, Sarstedt)

Electroporation cuvettes (4 mm gap) (Peqlab)

Nitrocellulose filter Hybond ECL™ (Amersham Biosciences)

ECL™ Western blotting detection reagents (Amersham Biosciences)

4.1.8 Instruments

Eppendorf Thermomixer comfort

Eppendorf centrifuge 5417R

pH meter inoLab® pH Level 1

Eppendorf BioPhotometer

Eppendorf Mastercycler personal and Eppendorf Mastercycler epgradient

Gel Imager (Intas, Göttingen)

Heraeus HERAcell 150 (cell culture incubators)

INFORS Unitron (37 °C shaker)

Heraeus Kelvitron®t (bacteria incubator)

Centrifuges SORVALL® RC 3B Plus and SORVALL® RC 26 Plus

T70/T77 semi-dry transfer unit (Hoefer)

Chemi Lux Imager (Intas, Göttingen)

HERAsafe (cell culture bench)

Heraeus Multifuge 3 S-R

BIO-RAD Gene Pulser & BIO-RAD Capacitance Extender

FACSCalibur (Becton Dickinson)

LSR II (Becton Dickinson)

4.1.9 Software

CellQuest; FlowJo (Treestar)

CSX-1400M Camera Controller (Chemi Lux Imager)

Gel documentation software GDS (Gel Imager)

Adobe® Photoshop® CS2 (image editing)

pDRAW 3.1; (*in silico* DNA analysis)

CorelDraw; MS Office

Endnote 7

4.2 Experimental Procedures

4.2.1 Methods in Molecular Biology

4.2.1.1 Digestion of DNA using restriction enzymes

For the sequence-specific cleavage of DNA molecules the purified DNA was incubated with restriction endonucleases according to the manufacturer's instructions.

4.2.1.2 Agarose gel electrophoresis of nucleic acids

Gels were prepared by dissolving 0.8-2% agarose (w/v) in TAE buffer. The agarose was melted in a microvave, ethidium bromide was added to a final concentration of 0.5 µg/ml, and the solution was poured into a sealed gel casting platform. The DNA samples to be resolved were mixed with an appropiate volume of 6x DNA loading buffer. Additionally a DNA molecular weight standard was loaded (GeneRuler 1 kb DNA ladder, MBI Fermentas). The electrophoresis was performed in a gel chamber filled with TAE buffer at a voltage of 5 V/cm distance between electrodes.

TAE-buffer.....	40 mM Tris-acetate, 1 mM EDTA
6x loading buffer.....	10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 60% glycerol

4.2.1.3 Extraction of DNA from agarose

Isolation of DNA fragments from agarose gels was achieved with Wizard SV Gel and PCR Clean-Up System (Promega) according to manufacturer's instructions.
DNA was eluted with 50µl of dH_2O.

4.2.1.4 Ethanol precipitation of DNA

DNA solutions were concentrated by ethanol precipitation by adding 1/10 volume 3 M NaOAc (pH5.2) followed by 2.5 volumes of ice-cold 100 % ethanol, and the samples

were then placed at -20 °C in a prechilled metal block for 5-10min. The samples were centrifuged at 16,000 x*g* at 4°C for 10 min. The supernatant was discarded and the DNA pellet was washed with 70% ethanol, centrifuged as before and the DNA pellet was air-dried. The dry pellet was dissolved in an appropriate volume of dH_2O or TE buffer, pH 8.0.

4.2.1.5 Photometric determination of DNA and RNA concentrations

DNA and RNA concentrations were determined using a BioPhotometer (Eppendorf) according to manufacturer's instructions. The DNA and RNA concentrations are calculated by the following formulas:

1 A_{260} Unit of dsDNA ≈ 50 µg/mL H_2O

1 A_{260} Unit of ssRNA ≈ 40 µg/mL H_2O

The purity of DNA or RNA can be estimated by the following values:

pure DNA: $A_{260}/A_{280} \geq 1.8$

pure RNA: $A_{260}/A_{280} \geq 2.0$

Low purity DNA (Minipreps) concentrations were further estimated by agarose gel electrophoresis.

4.2.1.6 Dephosphorylation of vector DNA

Prior to ligation, the vector DNA was dephosphorylated at the 5`-end with calf intestine phosphatase (CIP) (NEB). 0.5 µL CIP (1U/µL) was directly added to each 20 µL of restriction digest and incubated at 37°C for up to 1 h. After gel electrophoresis the dephosphorylated vector DNA was extracted from agarose (see 4.2.1.3).

4.2.1.7 Ligation of DNA fragments

For the ligation of linearized vectors and DNA fragments encoding proteins (inserts) T4 DNA ligase was used (NEB) according to manufacturer´s instructions. This enzyme catalyzes the formation of phosphodiester bonds between adjacent 3`-OH and 5`-PO_4 ends in dsDNA. For ligations a molar ratio of 1/3 (vector/insert DNA) was chosen. Usually 20ng of vector was subjected to ligation in a 10µl reaction. In order to check the ability of vector DNA to religate, one sample was set up without the addition of insert

DNA. Ligation reactions were incubated at 16°C overnight, and used directly for the transformation of competent bacteria.

4.2.1.8 Generation of transformation competent E. coli bacteria and transformation

5 ml LB medium were inoculated with the E. coli strain and incubated at 37°C overnight with shaking (180 rpm). The next day 150 ml LB medium were inoculated with 900 µl overnight culture. The culture was grown until an OD600 value of 0.45-0.55 was reached. The bacteria suspension was put on ice for 10 min and then harvested at 5,000 xg at 4°C for 10 min. The supernatant was removed and the bacteria were resuspended with 30 ml TFB I. After 10 min incubation on ice, the bacteria were centrifuged and resuspended in 6 ml ice-cold TFB II. 50 µl portions of this cell suspension were transferred to a 1.5 ml test tube and were flash frozen with liquid nitrogen. The competent bacteria were stored at -80°C until needed.

LB medium	10 g tryptone; 5 g Yeast Extract; 10 g NaCl ad 1000 mL
TFB I	50 mM $MnCl_2 \cdot 4\ H_2O$; 100 mM KCl; 10 mM $CaCl_2 \cdot 2\ H_2O$; 30 mM KOAc, pH 6.0; 15 % (v/v) glycerol; adjust to pH 6.1 with HOAc; autoclaved; 4 °C storage
TFB II	75 mM $CaCl_2 \cdot 2\ H_2O$; 10 mM KCl; 10 mM MOPS; 15 % (v/v) glycerol; adjust to pH 7.0 with KOH; autoclaved; 4 °C storage

Competent bacteria were thawed on ice. 10-50 ng of plasmid DNA or 5 µL of a ligation reaction were added to the bacteria and the suspension was mixed by gently flicking the tube. The transformation sample was incubated on ice for 15 min and then heat-shocked at 42°C for 60 s. The bacteria were returned to ice for 2 min and then 800 µL LB medium were added, and incubated at 37°C for 45 min. Subsequently, the bacteria were harvested at 6000 xg for 2 min, taken up in 150 µl LB medium and spread onto LB plates containing an appropriate antibiotic and incubated at 37°C overnight.

4.2.1.9 Isolation of Plasmid-DNA

For the analytical plasmid isolation (Miniprep) 5 ml LB medium supplemented with the appropriate antibiotic were inoculated with a single bacterial colony at 37°C in a shaker (180 rpm) overnight. The next day the plasmid DNA was isolated using the Wizard® Plus SV Minipreps Kit (Promega) according to manufacturer's instructions. For the production of larger amounts of plasmid DNA (Midiprep), 100-150 ml LB medium supplemented with the appropriate antibiotic were inoculated and the culture was grown at 37°C in a shaker (200 rpm) overnight to a density of OD600 2-4. The preparation of plasmid DNA was carried out with the NucleoBond PC 100 (Macherey-Nagel) according to manufacturer's instructions.

4.2.1.10 Isolation of total RNA from eukaryotic cells

Total RNA was isolated by using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Disruption and homogenization of cell lysates was carried out using QIAshredder columns (Qiagen) according to manufacturer's instructions. The isolated RNA was used directly for reverse transcription or stored at -80 °C.

4.2.1.11 Polymerase chain reaction:

The Polymerase chain reaction is a technique for the selective in vitro amplification of specific DNA fragments (Saiki et al., 1985; Mullis et al., 1986; Mullis and Faloona, 1987).

For amplification of chicken Btk cDNA and for introducing mutations, e.g. epitope tags, at the 5` or 3` end of a cDNA, PCR reactions were performed using the thermostable proofreading polymerase Pfu (Promega). Reactions were set up according to manufacturer's instructions.

4.2.1.12 Site directed mutagenesis:

Site directed mutagenesis was performed according to a published protocol (Fisher and Pei, 1997).

Briefly, two complementary synthetic oligonucleotides of 30-35 bases were utilized, containing the desired mutation in the middle. After a PCR reaction using Pfu DNA polymerase to amplify the complete plasmid the parental DNA is degraded by DpnI

(this enzyme only digests methylated DNA). The reaction is transformed into Top10F´ competent bacteria and plasmids from individual clones are analyzed for carrying the desired mutation.

4.2.1.13 Cloning of PCR products

PCR products generated with Pfu polymerase were purified using Wizard SV PCR clean up Kit (Promega) and incubated with Taq polymerase (NEB) at 72°C for 10min to add 3`-A overhangs. The reaction was set up like a standard PCR reaction according to the manufacturer´s recommendations.

Cloning of PCR products with 3'-A overhangs was carried out using the TOPO TA Cloning Kits from Invitrogen, using the pCR2.1-Topo vector. The ligation reactions were set up according to the manufacturer's instructions.

4.2.1.14 DNA sequence analysis

Sequencing was performed by MWG Biotech (Ebersberg, Germany).

4.2.2 Cell culture methods

4.2.2.1 Cell lines

DT40 (ATCC Number: CRL-2111)

The chicken DT40 B cell line is derived from an avian leucosis virus (ALV)-induced bursal lymphoma (Baba and Humphries, 1984; BABA et al., 1985). The original lymphoma was induced by viral infection of a 1 day old chicken with Rous-associated virus 1 (RAV-1). DT40 cells express surface IgM and continue to undergo IgL gene conversion during in vitro cell culture (Buerstedde et al., 1990; Kim et al., 1990). The most unique feature of DT40 cells is the high ratio of targeted to random integration of exogenous DNA. Targeted integration occurs in frequencies similar to those of random integration (Buerstedde and Takeda, 1991).

WEHI-231 (ATCC Number: CRL-2111)

WEHI-231 B cells derive from a mineral-oil induced tumor in (Balb/c x NZB) F1 mice. They express surface IgM.

Bal17.TR

Subclone of the IgM-containing Bal17 murine lymphoma, which has lost Grb2 expression for unknown reasons (Harmer and DeFranco, 1999).

4.2.2.2 Cell culture conditions

All used cell lines were cultured in a 5% CO_2 humidified atmosphere at 37°C. Wild-type DT40 B lymphocytes and derivatives were cultured in RPMI1640 containing 10% FCS, 1% CS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin. Murine B cell lines Bal17.TR and WEHI-231 were cultured in RPMI1640 containing 10% FCS, 2 mM L-glutamine, 1 mM pyruvate, 50 µM β-mercaptoethanol, 50 U/mL penicillin, and 50 µg/mL streptomycin. Plat-E cells were cultured in DMEM containing 10% FCS, 2 mM L-glutamine, 10 µg/mL blasticidin, 2.5 µg/ml puromycin, 50 U/mL penicillin, and 50 µg/mL streptomycin. The adherent growing Plat-E cells were split using Trypsin/EDTA solution after rinsing 1x with PBS.

4.2.2.3 Freezing and thawing of eukaryotic cells

For freezing, $1x10^7$ cells were harvested and resuspended in 1 ml of freezing medium (90% (v/v) FCS, 10% (v/v) DMSO). Vials were placed in a polystyrene box at -80°C for 24 h and then transfered to -150°C for long term storage.

Cells were thawed rapidly at 37°C and immediately washed in 10ml of culture medium. Cells were transfered in fresh culture medium into a cell culture dish and cultured as described above.

4.2.2.4 Transfection of DT40 lymphocytes by electroporation

$1 x10^7$ cells were harvested, washed with PBS, and resupended in 0.7 ml PBS. Then 25 µg of linearized DNA and the cell suspension were transferred to an electroporation cuvette (4 mm electrode gap, Peqlab Biotechnologie GmbH, Germany). After 10 min incubation on ice the cells were electroporated at 260 V and 960 µF . The electroporated cells were incubated on ice for 10 min and resuspended in DT40 medium. The cells

were cultured for 24 h and then resuspended in DT40 medium containing the appropriate selective antibiotic (see below). The cells were plated in two 96-well-dishes with 0.2 mL/well. After 7-10 days stable transfectants were visible and transferred to 24-well-dishes for further analysis.

Concentrations used:

300 µg/ml Zeocin or 30µg/ml Bleocin

50 µg/ml Blasticidin

2 mg/ml G418

1 µg/ml Puromycin

4.2.2.5 Transfection of Plat-E cells with FuGENE6 for the production of recombinant viruses

Plat-E cells were used as packaging cell line for the production of recombinant, replication incompetent retroviruses. These retroviruses were then used for the transduction of B lymphocytes. Plat-E cells were split 24 h prior to transfection to reach about 50-70% confluency at the time of transfection (6 cm dish).

The following components were mixed in the given order:

200µL RPMI w/o supplements

7.5 µL FuGENE6® (Roche Diagnostics) (directly pipetted into liquid)

1.9 µg retroviral expression vector (pMSCV or pLNCX2)

0.7 µg phCMV-VSV-G (for pseudotyping retroviruses, if DT40 chicken cells were to be transduced)

The components were mixed by tapping and left at RT for 30 min at RT. The Plat-E medium was aspirated from the dish and fresh DT40 medium was given onto the cells. The transfection mixture was added dropwise to the cells. After 48h incubation at 5% CO_2 and 37°C, the cell supernatant containing the retroviruses was used for transduction.

4.2.2.6 Transfection of lymphocytes by retroviral gene transfer

The retroviral gene transfer technology introduces efficiently stable, heritable genetic material into the genome of any dividing cell type. The retroviral gene transfer

technology is based on the parallel design of packaging cell lines and retroviral expression vectors. Packaging cell lines package recombinant retroviral RNAs into infectious, replication-incompetent particles. In a packaging cell line, the viral *gag*, *pol*, and *env* genes are stably integrated into the genome. Transfection of the retroviral vector into a packaging cell line produces replication-incompetent viruses, with the viral genomic transcript containing the target gene and the selectable marker. The viral *env* gene expressed by the packaging cell line encodes the envelope protein. This protein determines the range of infectivity (tropism) of the packaged virus.

4.2.2.7 Transfection of DT40 cells with recombinant retroviruses

The retrovirus-containing supernatant of the transfected PlatE cells was sterile filtered using 0.45 µm pore size and applied to a new 6cm cell culture dish. $1x10^6$ DT40 or Bal17.TR cell were suspended in 1.5ml of fresh medium and mixed with the retrovirus-containing supernatant. Polybrene from a freshly prepared stock solution (3 mg/ml in dH2O) was added to a concentration of 3 µg/ml. Cells were incubated for 24h under standard cell culture conditions. To remove remaining retrovirus, the cells were centrifuged and resuspended in 5 ml of fresh medium. 24h later the appropriate selective antibiotic was added to the cells.

All manipulations of pseudo-typed retroviruses were performed incompliance with the S2 standard and safety instructions.

4.2.2.8 Ca^{2+} mobilization analysis

The Ca^{2+} response in B lymphocytes after stimulation was determined by flow cytometry using the Ca^{2+}-sensitive fluorophor Indo-1. The acetoxymethyl ester derivative Indo-1 (Indo-1-AM) is hydrophobic molecule that is capable of permeating cell membranes. Within the cell, the lipophilic blocking group is hydrolized by cytoplasmic esterases, resulting in a charged form which can permeate the plasma membrane far more slowly than its parent compound (“dye-trapping”).

Indo-1 is a ratiometric dye, i.e. the emission shifts from about 475 nm (blue) without Ca^{2+} to about 400 nm (violet) when complexed with Ca^{2+}. Excitation maximum is at about 350 nm. The use of the 400/475 emission ratio considerably reduces the effects of unequal dye loading, leakage of dye, photobleaching, and problems associated with

measuring Ca^{2+} in cells of unequal size/shape. For Ca^{2+} mobilization analysis, 2 x10^6 cells were harvested at 300x*g* and 4°C for 5 min. The cell pellet was resuspended in 1ml of the corresponding cell culture medium and Indo-1-AM was added to 1 µM. Cells were incubated 30min at 30°C and the 10min at 37°C. The cells were subsequently washed 1x with Krebs-Ringer solution and resuspended in 1 ml of Krebs-Ringer solution. Cells were then kept at 25°C until measurement but at least 15 min. If the intracellular Ca^{2+} level was to be analyzed independent of Ca^{2+} influx across the plasma membrane, the cells were harvested and resuspended in Krebs-Ringer solution containing EGTA instead of Ca^{2+} just prior to the measurement. Kinetic analysis of emission of Indo-1 at 385-425 nm (violet) and 500-560 nm (blue) was performed with a LSRII flow cytometer (Becton Dickinson).After taking a 30 s baseline cells were removed from the instrument, stimulated with BCR crosslinking antibodies as indicated in each figure and put back to the instrument. Kinetic graphs in each figure represent the median of all cells analyzed in a given second. Each experiment was performed at least 3 times.

Krebs-Ringer solution: 10 mM HEPES, pH 7.0; 140 mM NaCl; 4 mM KCl; 1 mM $MgCl_2$; 10 mM glucose

Ca^{2+}-containing: + 1 mM $CaCl_2$

Ca^{2+}-free/EGTA-containing: + 0.5 mM EGTA

Indo-1-AM stock: 1 mM in DMSO; store at -20 °C

4.2.3 Biochemical Methods

4.2.3.1 Stimulation of B lymphocytes via the BCR

B cells were harvested and washed once with RPMI 1640 medium without supplements. The cells were resuspended in RPMI 1640 medium without supplements at a density of 2-4 x10^7 cells/ml and incubated at 37°C for 10-25 min. For stimulation of B cells via BCR alone or BCR + FcγRIIb the following crosslinking antibodies were added in the given order:

DT40:	
BCR stimulation	2 µg/ml M4
BCR + FcγRIIb crosslinking	15 µg/ml IgG rabbit anti-mouse IgM 2 µg/ml M4
BCR stimulation (when compared to FcγRIIb co-ligation)	10 µg/ml $F(ab)_2$ goat anti-mouse IgM 2 µg/ml M4
WEHI231 / Bal17.TR:	
BCR stimulation	10µg/ml $F(ab)_2$ goat anti-mouse IgM
BCR + FcγRIIb crosslinking	15µg/ml IgG rabbit anti-mouse IgM

After addition of the crosslinking antibodies the samples were vortexed and incubated at 37°C for the indicated time. Reactions were stopped by placing on ice. Cells were harvested at 1000xg for 1 min at 4°C and the cell pellet was lysed in 250µl lysis buffer per 1 $x10^7$ cells. After 10 min incubation on ice the insoluble debris was removed by centrifugation for 10min at maximum speed at 4°C. The supernatant containing soluble material (cleared cellular lysate (CCL)) was transferred to a fresh tube. The CCLs were either directly mixed with 1/3 of 4x SDS sample buffer or used for immunoprecipitations.

lysis buffer ..... TBS; 1% NP40; 5 mM NaF; 1 mM Na_3VO_4; protease inhibitor cocktail

4x SDS sample buffer ..... 250 mM Tris/HCl, pH 6.8; 200 mM DTT; 40% glycerol; 8 % SDS; 0.05% bromophenol blue

4.2.3.2 Affinity purification and imunoprecipitation

Affinity purification of proteins from CCLs was performed with GST-fusion protein containing the PTB domain of chicken Dok-3 and a mutant version (R297A) (in cooperation with M. Lösing). A 2 ml aliquot of the GST fusion protein was thawed on ice and centrifuged at maximum speed and 4 °C for 10 min. Glutathione Sepharose 4B beads (Amersham Biosciences) were added to the supernatant and rotated at 4 °C for at

least 1 h (binding capacity ~10 mg recombinant GST/ml medium). The beads were washed three times with lysis buffer (4.2.3.1). 10-20 µl beads were added to the CCLs of 2 x10^7 cells and rotated at 4 °C for at least 1 h. Then the beads were washed three times with lysis buffer (see 4.2.3.3.). Subsequently 40 µl 2x SDS sample buffer were added and the samples were heated to 95°C for 5 min. Purified proteins were resolved by SDS-PAGE.

For immunoprecipitations of proteins from CCLs, 0.5-2 µg of the corresponding antibodies were added to the lysates of 1-2 x10^7 cells and rotated at 4°C for at least 1 h. Then 15-20 µl Protein A/G Agarose beads (Santa Cruz or Pierce) were added and protein complexes were captured while rotating at 4°C for 1h. Subsequently, the steps as described for affinity purifications were followed.

4.2.3.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Separation of proteins was carried out based principally on the method of one-dimensional, discontinuous electrophoresis in sodium dodecyl sulphate (Laemmli, 1970). The final acrylamide concentration was 8-12.5 % for separating gels, and 4.8% for stacking gels. Prestained Protein Marker (6.5-175 kDa, New England Biolabs) was used as standard. The electrophoretic separation was carried out at a constant current of 10 mA for stacking gels and 25 mA for separating gels. Subsequently gels were subjected or western blot analysis:

separating gel	375 mM Tris/HCl, pH 8.8; 8-12.5% acrylamide; 0.1% SDS; 0.1% TEMED; 0.1% APS
stacking gel	125 mM Tris/HCl, pH 6.8; 4.8% acrylamide; 0.1% SDS; 0.1 % TEMED; 0.1 % APS
SDS running buffer	25 mM Tris; 192 mM glycine; 0.1% (w/v) SDS

4.2.3.4 Western blotting

Western blotting was performed with a semidry transfer unit (Hoefer) according to the manufacturer´s instructions. Briefly, a sheet of transfer buffer-soaked filter paper was put in place on the anode. The filter paper was followed by the equilibrated nitrocellulose membrane, the gel and finally another sheet of transfer buffer-saturated

filter paper. Air bubbles were rolled out as each component was added to the stack. The cathode was placed atop of the transfer stack and a current of max. 0.8 mA/cm^2 gel was applied for 1 h. After transfer the unit was disassembled and the membrane with transferred proteins was subjected to immunoprobing with primary and secondary horseradish-peroxidase (HRPO)-conjugated antibodies. The membrane was first incubated in blocking solution for 1 h at 4°C overnight on a rocking platform, and thereafter washed three times for 5 min with TBS-T. The membrane was incubated with the primary antibody in blocking solution at 4°C overnight on a rocking platform. The membrane was washed with TBS-T three times for 5 min. The HRPO-conjugated anti-immunoglobulin antibody was diluted 1/10,000 in TBS-T, and the membrane was incubated with the secondary antibody at 4°C for 1 h on a rocking platform. The membrane was washed with TBS-T four times for 10 min with mild agitation. The immunoblot was visualized using the ECL detection system (Amersham Biosciences). Detection of specific proteins was carried out by exposure of the membrane to a digital imaging system (Chemi Lux Imager, Intas).

Transfer buffer	39 mM glycine; 48 mM Tris; 0.0375% (w/v) SDS; 0.01% (w/v) NaN_3; 20% MeOH
Blocking solution	5% (w/v) BSA in TBS-T
TBS-T	0.1% (v/v) Tween-20 in TBS

5 Results

5.1 Grb2 and Dok-3 inhibit Ca^{2+} release from the ER Ca^{2+} stores

BCR-induced Ca^{2+} mobilization occurs in two phases. In the first phase, the binding of IP3 to its receptors in the ER membrane results in release of Ca^{2+} from ER stores. The depletion of the Ca^{2+} stores leads to the second phase of sustained influx of extracellular Ca^{2+} across the plasma membrane.

Studies using B cells deficient for the cytoplasmic adaptor protein Grb2 have shown that it inhibits Ca^{2+} mobilization in response to BCR stimulation (Stork et al., 2004). During my work on the molecular mechanism of this observation, Björn Stork in our laboratory identified the adaptor protein Dok-3 as a Grb2 binding partner that also inhibits Ca^{2+} mobilization (Stork, 2006). To test, how both of these proteins inhibit the rise in cytosolic Ca^{2+} in response to BCR crosslinking, their impact on the two phases of Ca^{2+} mobilization were investigated. As shown in Figure 3A, DT40 cells rendered deficient for Dok-3 or Grb2 expression show an increased rise in cytosolic Ca^{2+} concentration in both phases, release of Ca^{2+} from the ER and Ca^{2+} influx across the plasma membrane (provided by B. Stork). To monitor the relative cytosolic Ca^{2+} concentration, cells were loaded with the fluorescent Ca^{2+} indicator INDO-1 and analyzed by flow cytometry. To discriminate between the two phases of Ca^{2+} mobilization cells were stimulated in the absence of extracellular Ca^{2+} to prevent Ca^{2+} entry. After the signal terminated, Ca^{2+} was restored to 1 mM to allow for Ca^{2+} entry.

The rise in the concentration of cytosolic Ca^{2+} in response to BCR ligation is due to IP3-mediated depletion of the ER Ca^{2+} stores which then leads to opening of CRAC channels of the plasma membrane. Yet, there are other mechanisms by which the cytosolic Ca^{2+} concentration can be altered, e.g., activated Ca^{2+} pumps or Ca^{2+} exchanger can extrude Ca^{2+} to the extracellular space and thus limit cytosolic Ca^{2+} rises. Blocking of Ca^{2+} extrusion would phenotypically also lead to increased Ca^{2+} concentrations in both phases of BCR-induced Ca^{2+} mobilization (Chen et al., 2004).

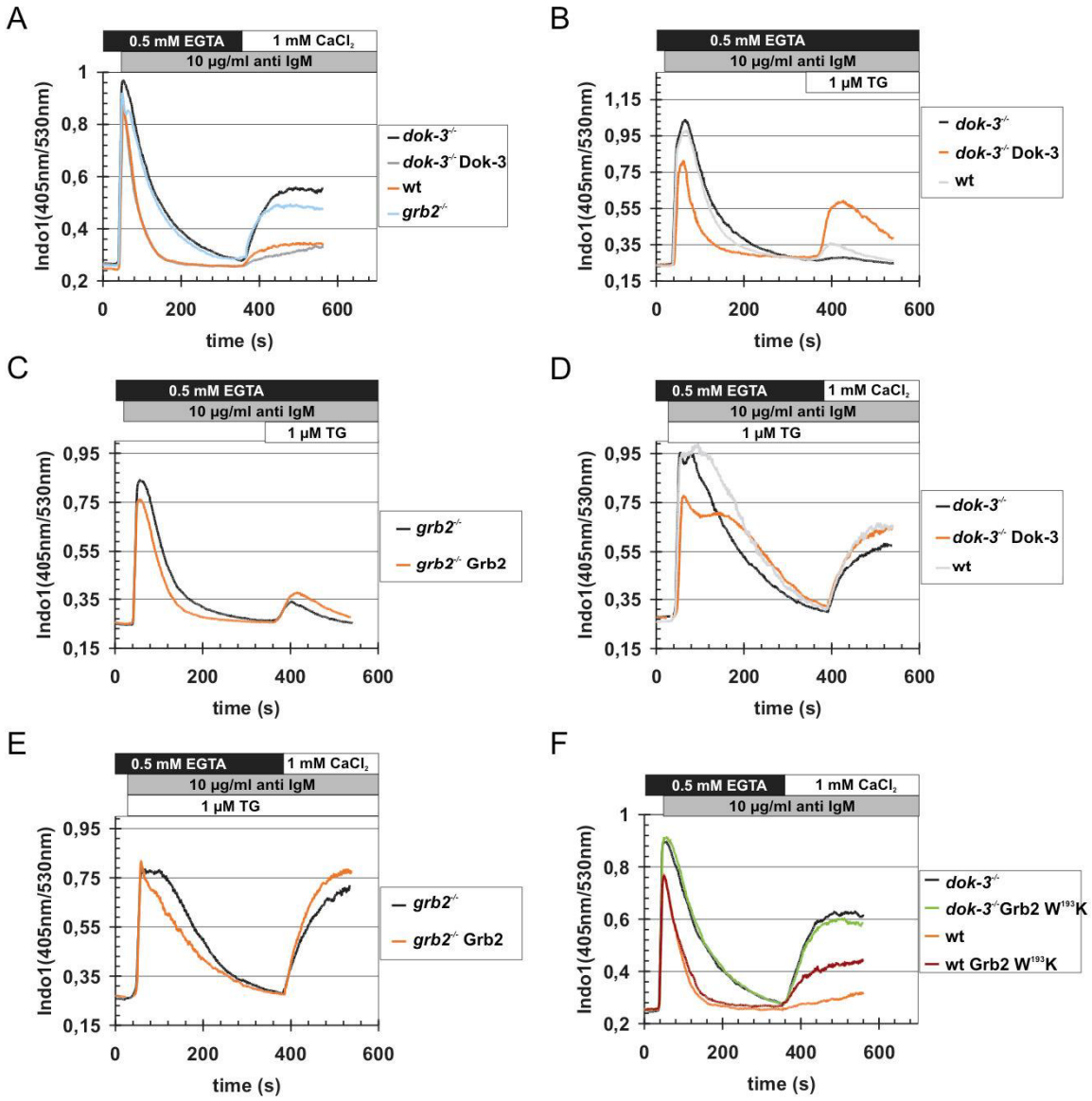

Figure 3: Dok-3 and Grb2 inhibit Ca^{2+} mobilization in B cells.
(A,F) *BCR-induced intra- and extracellular Ca^{2+} mobilization of the indicated DT40 cells was recorded by flow cytometry as described in detail in Materials and methods. Briefly, cells were loaded with Indo-1 and release of intracellular Ca^{2+} was measured for 6 min in the presence of EGTA. Subsequently, extracellular Ca^{2+} was restored to 1mM 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), grb$2^{-/-}$ mutants (blue) and wild-type and dok-$3^{-/-}$ transfectants expressing the dominant-negative $W^{193}K$ version of Grb2 (brown and green, respectively).*
(B,C) *Ca^{2+} remaining in the ER of indicated DT40 cells was determined by stimulating the cells in the presence of EGTA. Ca^{2+} remaining in the ER Ca^{2+} stores released after 6 min by addition of 1µM Thapsigargin (TG)*
(D,E) *Ca^{2+} entry with completely depleted ER Ca^{2+} stores. Indicated DT40 cells were stimulated by addition of anti-IgM and Thapsigargin in the presence of EGTA. After 6 min extracellular Ca^{2+} was restored to 1mM in order to monitor Ca^{2+} entry across the plasma membrane.*

To asses, whether both Grb2 and Dok-3 indeed inhibit the release of Ca^{2+} from the ER, the amount of Ca^{2+} remaining in the ER after the first phase of Ca^{2+} release was determined in DT40 cells deficient for either Grb2 or Dok-3. The cells were stimulated via the BCR in the absence of extracellular Ca^{2+} and after the signal terminated, Ca^{2+} remaining in the ER was released to the cytosol by addition of Thapsigargin. Thapsigargin is an inhibitor of the ER membrane Ca^{2+} pump SERCA, which pumps Ca^{2+} from the cytosol into the ER. Inhibition of SERCA leads to passive diffusion of Ca^{2+} from the ER into the cytosol, which can be monitored by flow cytometry. As shown in Figure 3 B & C, in cells deficient for Grb2 or Dok-3 significantly less Ca^{2+} remains in the ER after the BCR-induced Ca^{2+} signal terminated. This shows that cells deficient for Grb2 or Dok-3 release more Ca^{2+} from the ER in response to BCR stimulation, i.e. both adaptor proteins inhibit Ca^{2+} release from the ER. Thus, the strong Ca^{2+} entry across the plasma membrane, observed in both Dok-3 and Grb2 deficient cells, could merely be due to a stronger degree of depletion of the ER Ca^{2+} stores, which induces opening of channels in the plasma membrane. Grb2 and Dok-3 could, however, also influence Ca^{2+} entry by directly regulating CRAC channels. To test this, the Ca^{2+} influx across the plasma membrane was measured after BCR stimulation of the cells in the presence of Thapsigargin, which completely depletes the Ca^{2+} from the ER. In this experimental setup, the Ca^{2+} influx is uncoupled from BCR-mediated store depletion, since the stores are completely depleted for each cell line. As shown in Figure 3 D & E, both Grb2 and Dok-3-deficient cells show similar Ca^{2+} entry as compared to their reconstituted counterparts in this experimental setup. This supports the notion that both adaptor proteins selectively inhibit Ca^{2+} release from internal stores, which then consequently inhibits store-operated CRAC channel opening.

As Björn Stork showed during my work, Grb2 binds to and is required for Dok-3 phosphorylation in response to BCR ligation. Thus, it was feasible to propose Dok-3 and Grb2 to constitute one signaling module together. To test this notion, a dominant-negative Grb2 mutant was employed, which harbors an inactivated C-terminal SH3 domain (W193K). As the C-terminal SH3 domain of Grb2 is essential for inhibition of Ca^{2+} mobilization in response to BCR stimulation (Stork et al., 2004), expression of Grb2 W193K in DT40 cells overwrites the inhibitory function of endogenous wild-type Grb2 and allows for stronger Ca^{2+} flux (Figure 3 F). In contrast, expression of the Grb2

W193K protein in Dok-3-deficient DT40 cells has no effect on the Ca^{2+} profile, indicating that Dok-3 expression is required for the inhibitory function of Grb2. This supports the notion that Dok-3 and Grb2 constitute a functional unit to attenuate BCR-induced Ca^{2+} mobilization.

5.2 Grb2 and Dok-3 inhibit BCR-induced activation of PLC-γ2

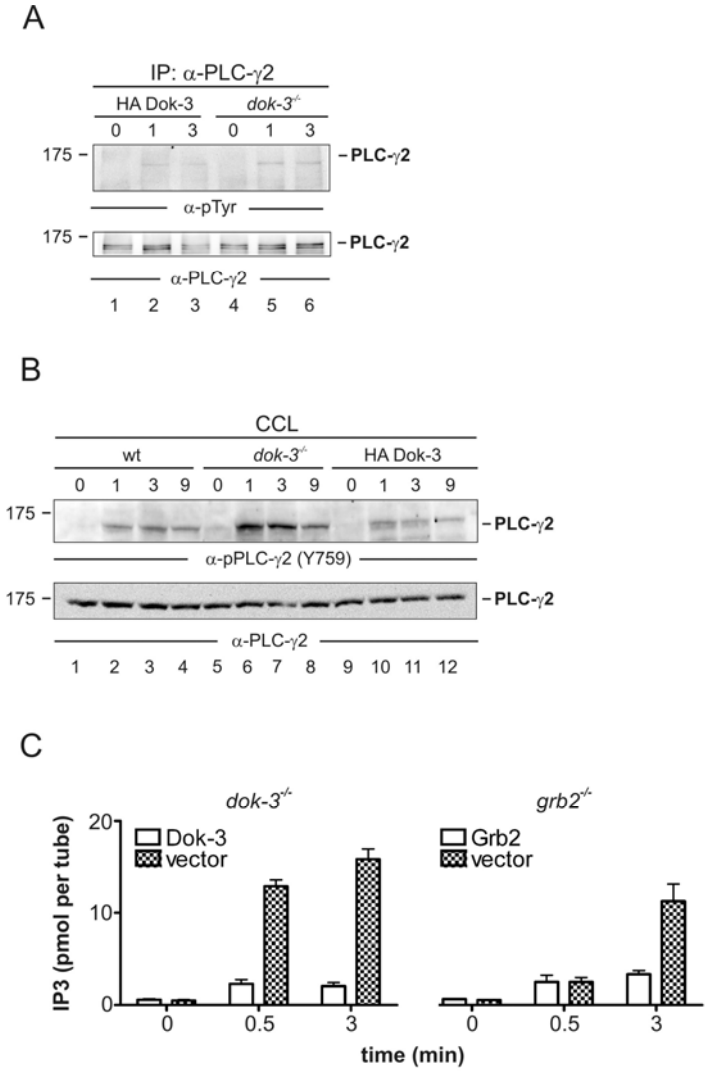

Figure 4: The Dok-3/Grb2 module attenuates PLC-γ2 activity.
(A) *Dok-3-deficient DT40 mutants (lanes 4–6) and reconstituted cells expressing HA-tagged wild-type Dok-3 (lanes 1–3) were left untreated (0) or stimulated through their BCRs for the indicated times (min). Lysates were subjected to anti-PLC-γ2 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 Y759 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 (2x10^6 cells per sample) 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.*

The release of Ca^{2+} from the ER into the cytosol in response to BCR ligation is mediated by IP3 produced by PLC-γ2, which is activated by membrane recruitment and subsequent phosphorylation by Btk in the linker region at tyrosines 753 and 759 (Kim et al., 2004). To assess the activation of PLC-γ2, the phosphorylation status of PLC-γ2 in Dok-3-deficient cells was assessed by immunoprecipitation of PLC-γ2 and subsequent Western-blot analysis using an anti-phospho-tyrosine antibody. As shown in Figure 4A, overall tyrosine phosphorylation of PLC-γ2 is only slightly increased in Dok-3-deficient cells. To more specifically assess phosphorylation of tyrosines required for activation, a phospho-specific antibody, recognizing Y759 of human PLC-γ2 only in its phosphorylated form, was used. This analysis shows a drastic increase of phosphorylation of this motif in Dok-3-deficient cells (Figure 4B). The kinetic and extent of PLC-γ2 phosphorylation at this specific residue was substantially upregulated in *dok3*$^{-/-}$ cells (lanes 5-8) compared with wild-type parental cells (lanes 1-4) or Dok-3-reconstituted transfectants (lanes 9-12). Since phosphorylation of this residue by Btk has been shown to activate PLC-γ2 catalytic activity (Kim et al., 2004), the product of PLC-γ2, namely IP3, was determined in Dok-3-deficient cells. Indeed, IP3 levels were strongly increased in BCR-stimulated *dok3*$^{-/-}$ cells compared to reconstituted transfectants (Figure 4C, left panel). The same was also true for *grb2*$^{-/-}$ cells (Figure 4C, right panel). The result that Grb2 inhibits IP3 production appeared to be in conflict with previously published data, showing no difference in global PLC-γ2 phosphorylation in response to BCR ligation in Grb2-deficient DT40 cells (Stork et al., 2004). The specific analysis of Y^{759} phosphorylation of PLC-γ2 did not succeed using these cells due to a low signal to noise ratio. To increase PLC-γ2 phosphorylation, Btk was overexpressed in Grb2-deficient cells, as phosphorylation by Btk is critical for activation of PLC-γ2 (Kim et al., 2004; Takata and Kurosaki, 1996). Overexpression of Btk in *grb2*$^{-/-}$ cells increased BCR-induced PLC-γ2 phosphorylation (Figure 5A, compare lanes 4 and 8). Reconstitution of Grb2 expression reduces total PLC-γ2 phosphorylation in these Btk-overexpressing cells (Figure 5A, compare lanes 6 and 8). This indicates that Grb2 inhibits Btk-mediated PLC-γ2 phosphorylation, and that the effect is merely too small to be visible in wild-type cells. In Btk-overexpressing cells it was also possible to analyze specifically phosphorylation of PLC-γ2 at Y759. Similar to the total phosphorylation of

the enzyme, the phosphorylation of Y759 was drastically increased in Grb2-deficient cells, as compared to reconstituted counterparts (Figure 5B, lanes 8 and 6, respectively). Together, these data show that both Grb2 and Dok-3 inhibit Ca^{2+} flux in response to BCR ligation by inhibiting activation of PLC-γ2 by Btk.

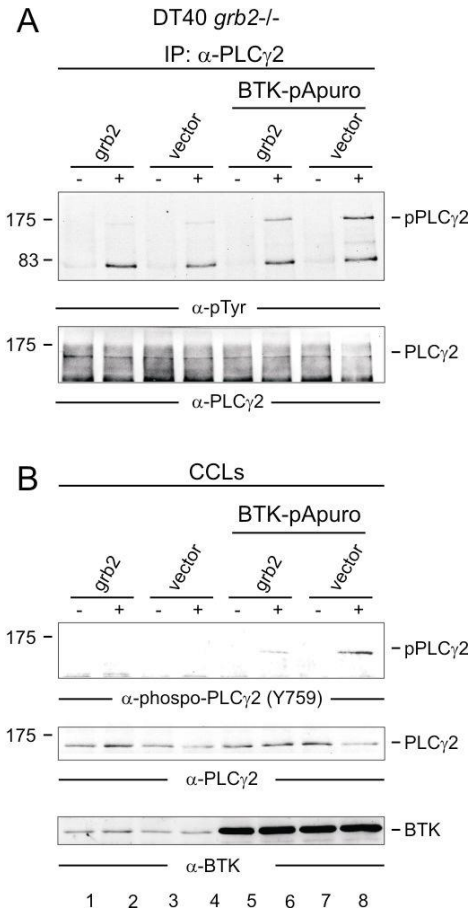

Figure 5: Grb2 inhibits Btk-mediated PLC-γ2 phosphorylation
(A) *Grb2-deficient DT40 cells, either untransfected (lanes 1–4) or overexpressing Btk (lanes5-8), were reconstituted with Grb2 or empty vector and were left untreated (-) or stimulated through their BCRs for 3min (+). Lysates were subjected to anti-PLC-γ2 immunopurification and proteins obtained were analyzed by anti-pTyr and anti-PLC-γ2 immunoblotting (upper and lower panels, respectively).*
(B) *Lysates from A were subjected to immunoblot analysis with antibodies that specifically detect PLC-γ2 phosphorylation at the Btk-dependent phospho-acceptor site corresponding to Y759 in human PLC-γ2 (upper panel). Equal protein loading was confirmed by reprobing the membrane with anti-PLC-γ2 antibodies (middle panel) and Btk expression was analyzed by anti-Btk immunoblotting (lower panel).*

5.3 Dok-3 acts independently of SHIP and CSK

The phenotype of Dok-3 and Grb2-deficient DT40 cells closely resembles the phenotype of SHIP-deficient DT40 cells, as SHIP also inhibits Btk-mediated PLC-γ2 activation (Bolland et al., 1998; Hashimoto et al., 1999). Further, Dok-3 and Grb2 have both been shown to bind to SHIP in BCR stimulated B cells (Harmer and DeFranco, 1999; Robson et al., 2004). To elucidate, whether the interaction with this negative regulator is responsible for the inhibitory function of the Grb2/Dok-3 module, mutants of Dok-3 were generated. In Dok-3-deficient cells reconstituted with these mutants, the ability of these mutants to inhibit Ca^{2+} flux was compared to their ability to bind to SHIP (The generation and analysis of the transfectants was done in cooperation with Ingo Goldbeck, Björn Stork and Michael Engelke). As shown in Figure 6B, deletion of

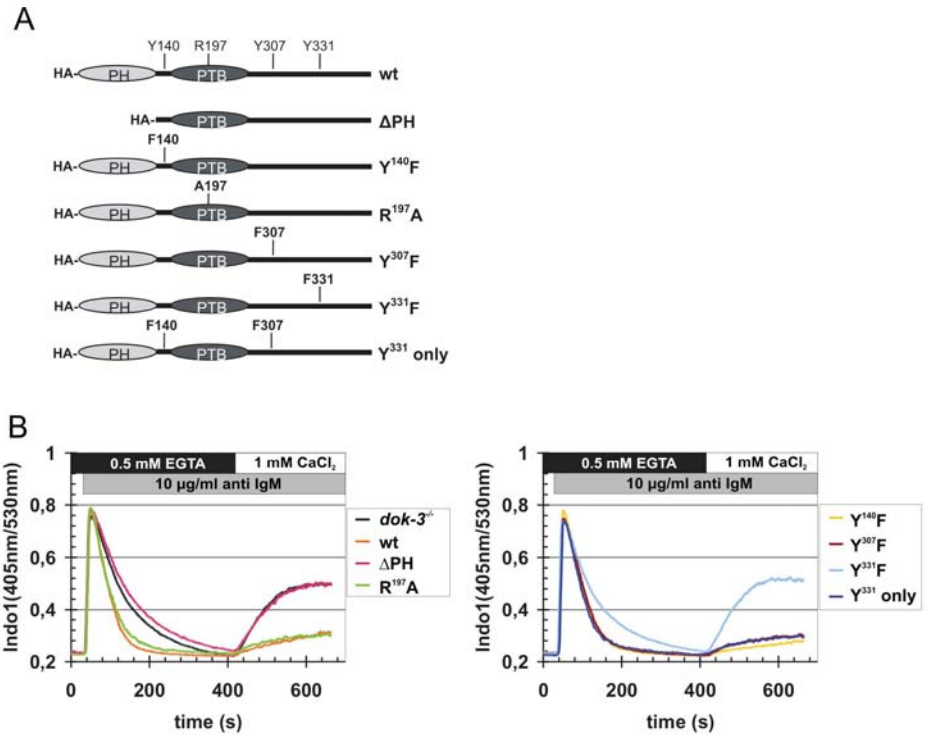

Figure 6: Only PH-domain and Grb2-binding site are required for Dok-3-mediated inhibition of Ca^{2+}-flux.. **(A)** *Schematic representation of expression constructs encoding HA-tagged versions of wild-type Dok-3, a PH domain deletion mutant (ΔPH) or mutants encompassing amino-acid exchanges depicted 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 3. Wild-type DT40 cells and empty vector transfectants of dok-$3^{-/-}$ mutants served as control (see inlay for color code)*

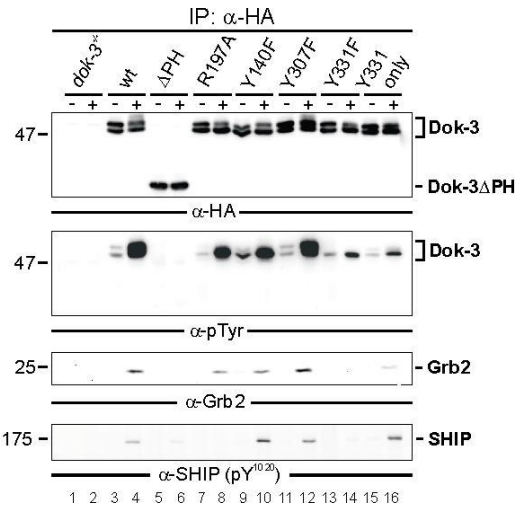

Figure 7: Dok-3 binds to Grb2 via Y331 and to SHIP via its PTB domain..
Wild-type and DT40 variants described in Figure 6 were left untreated (−) or BCR-activated (+) and lysates were subjected to anti-HA immunoprecipitation. Expression and tyrosine phosphorylation of Dok-3 proteins, as well as their association to Grb2 and SHIP, were detected by sequential immunoblotting with antibodies to HA, pTyr, Grb2 and SHIP (upper to lower panels, respectively).

the N-terminal PH-domain abrogates inhibition of Ca^{2+} mobilization by Dok-3. The association of this mutant with SHIP is also abrogated, as determined by co-immunoprecipitation experiments (Figure 7, lanes 5 and 6). This was shown to be due to impaired membrane recruitment of Dok-3 (Goldbeck, 2007). Inactivation of the PTB domain of Dok-3 by introducing an amino acid exchange ($R^{197}A$) does not interfere with Dok-3-mediated inhibition of Ca^{2+} mobilization (Figure 6B). Strikingly, this mutant does not co-precipitate SHIP (Figure 7, lanes 7 and 8). This indicates that the interaction between Dok-3 and SHIP is dispensable for Dok-3-mediated inhibition of Ca^{2+} flux in response to BCR ligation.

There are three putative tyrosine phosphorylation sites in Dok-3. Y^{331} is essential for binding to Grb2, phosphorylation of Dok-3 and inhibition of Ca^{2+} mobilization by Dok-3 (Stork, 2006). Mutation of this residue also abrogates binding to SHIP (Figure 7, lanes 13 and 14). Y^{140} of Dok-3 is dispensable for both the function and binding to SHIP. Y^{307} of Dok-3 has been shown to be essential for binding to C-terminal Src kinase (CSK) (Robson et al., 2004). Mutation of Y^{307} does not impede Dok-3-mediated

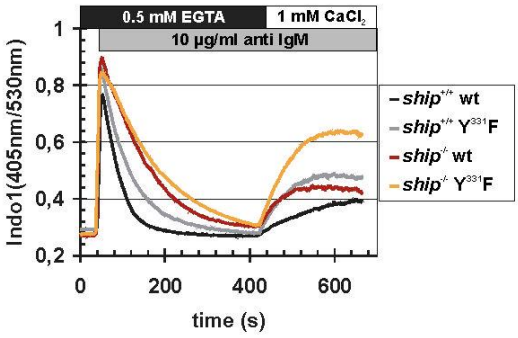

Figure 8: Dominant-negative mutant of Dok-3 acts in the absence of SHIP. BCR-induced Ca^{2+} fluxes were analyzed as described in the legend to Figure 3 in SHIP-deficient DT40 cells ($ship^{-/-}$, brown line) and $ship^{-/-}$ transfectants expressing a Dok-3 $Y^{331}F$ variant that counteracts Ca^{2+} inhibition by endogenous wild-type Dok-3 (orange line). As control, parental DT40 cells, which are positive for endogenous SHIP and Dok-3 (black line), and the Dok-3 $Y^{331}F$ transfectants (gray) were analyzed in parallel, demonstrating the dominant-negative function of Dok-3 $Y^{331}F$.

inhibition of Ca^{2+} mobilization (Figure 7C). This suggests that the interaction of CSK and Dok-3 is also dispensable for Dok-3 function.

To further analyze if Dok-3 can function independently of SHIP, a dominant-negative mutant of Dok-3 was expressed in SHIP-deficient DT40 cells. The Dok-3 mutant harboring a $Y^{331}F$ mutation is unable to bind to Grb2 and to inhibit Ca^{2+} flux. Expression of this mutant induces increased Ca^{2+} mobilization in DT40 wild-type cells, probably by competing with endogenous Dok-3 (Figure 8). Similarly, expression of this mutant also induces increase in Ca^{2+} mobilization in DT40 cells deficient for SHIP, which means that the endogenous Dok-3 inhibits Ca^{2+} mobilization in the absence of SHIP. This finding further supports the notion that the negative role of Dok-3 in BCR signaling is independent of SHIP.

5.4 Dok-3 does not influence SHIP and CSK activity

As described in the former section, mutational analysis of Dok-3 has revealed that Dok-3 can function independent of SHIP and CSK in DT40 cells. To test, whether Dok-3 influences the properties of the two proteins, their activity was assayed in Dok-3-deficient cells.

As CSK phosphorylates Src-kinases at their C-terminal inhibitory tyrosine and Lyn is the only Src-kinase expressed in DT40 cells, the phosphorylation state of both the activatory tyrosine (Y416 of human Lyn) and the inhibitory tyrosine (Y507 of human Lyn) was determined using antibodies recognizing these two sites, only when phosphorylated. No differences in phosphorylation status of Lyn at any of the two sites

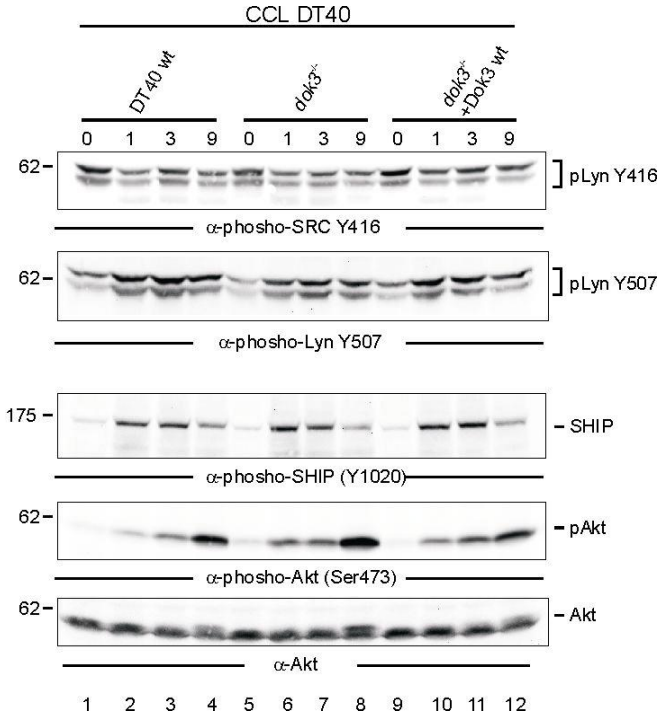

Figure 9: Lyn and SHIP activation is intact in the absence of Dok-3 expression. Wild-type DT40 cells (lanes 1-4), dok-3-/- mutants (lanes 5-8) and their reconstituted transfectants expressing HA-tagged Dok-3 (lanes 9-12) were left untreated (0) or stimulated through their BCR for the indicated times (min). Cleared cellular lysates (CCL) were analyzed by immunoblotting with site-specific anti-pTyr antibodies to the activating auto-phosphorylation site of Lyn (Y416) (upper panel) and the inhibitory pTyr residue 507 (second panel), which is the direct substrate of Csk.
To tyrosine-phophorylated SHIP (Y1020), serine phosphorylated Akt (S473) and total Akt (third, fourth and lower panel, respectively). Relative molecular masses of marker proteins are indicated on the left in kDa. As the presence or absence of Dok-3 expression causes little or no differences in the phosphorylation status of the investigated enzymes and/or their downstream effectors, neither SHIP nor Csk appear to be a major target of Dok-3.

can be observed in Dok-3-deficient cells in response to BCR stimulation (lanes 5-8), compared to wild-type parental cells (lanes 1-4) or Dok-3-reconstituted transfectants (lanes 9-12) (Figure 9, first and second panel). This again shows no involvement of CSK in Dok-3 function downstream of the BCR.

The phosphorylation of SHIP in response to BCR stimulation was determined by Western blot analysis using an antibody recognizing SHIP only when phosphorylated at Y1020 (of human SHIP). This tyrosine residue has previously been shown to be important for SHIP-mediated inhibition of signaling (Sattler et al., 2001). As shown in Figure 9 (middle panel), BCR-induced phosphorylation of SHIP at Y1020 is not altered in Dok-3-deficient cells (lanes 5-8) compared to wild-type parental cells (lanes 1-4) or Dok-3-reconstituted transfectants (lanes 9-12). Further, phosphorylation of Akt (also called PKB) at S473, which depends on the presence of the PI3K product PI(3,4,5)P3 and therefore is inhibited by SHIP (Aman et al., 1998), is hardly altered (Figure 9,

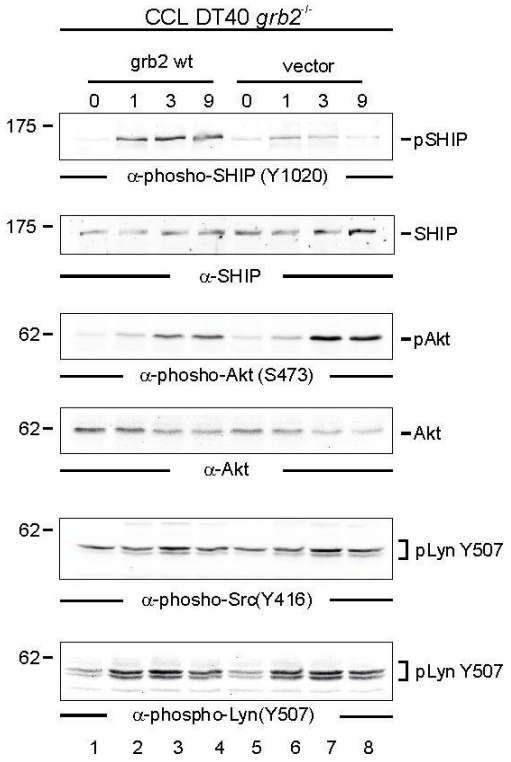

Figure 10: Grb2-deficiency leads to reduced SHIP activation
DT40 grb2-/- mutants were transfected with wild-type Grb2 (lanes1-4) or empty vector (5-8) were left untreated (0) or stimulated through their BCR for the indicated times (min). Cleared cellular lysates (CCL) were analyzed by immunoblotting with anti-pTyr antibodies to tyrosine-phophorylated SHIP (Y1020), serinephosphorylated Akt (S473) and total SHIP and Akt (first, third, second and fourth panel, respectively). the activating auto-phosphorylation site of Lyn (Y416) (second lowest panel) and the inhibitory pTyr residue 507 (lowest panel), which is the direct substrate of Csk. Relative molecular masses of marker proteins are indicated on the left in kDa.

second lowest panel). Together, this indicates that SHIP is not the target of Dok-3-mediated inhibition.

In contrast, phosphorylation of SHIP at Y1020 is reduced in *grb2-/-* DT40 cells in response to BCR ligation (Figure 10, first panel, lanes 5-8) compared to Grb2-reconstituted transfectants (lanes 1-4). Consequently, phosphorylation of Akt at S473 is enhanced in the absence of Grb2 (third panel). This suggests that Grb2 enhances SHIP activation and thus inhibits BCR signaling also by additional Dok-3-independent mechanisms. The phosphorylation state of Lyn at both activatory and inhibitory tyrosine residues is not altered in Grb2-deficient cells (Figure 10, second lowest and lowest panel, respectively). Collectively, the data show that Grb2 and Dok-3 constitute a signaling module that inhibits PLC-γ2 activation and subsequent Ca^{2+} mobilization by a SHIP and CSK-independent mechanism, while Grb2 seems to have additional Dok-3-independent functions.

5.5 Dok-3 tyrosine phosphorylation is enhanced by FcγRIIb coligation

As described in the previous section, the inhibitory function of Dok-3 seems to be independent of SHIP. Yet, the binding of Dok-3 to SHIP has previously been shown in mouse B cells (Robson et al., 2004) and could also be shown in the DT40 system (Figure 7). If the interaction is of functional significance, SHIP might rather be important for Dok-3 activation than *vice versa*. SHIP is only weakly phosphorylated in B cells stimulated via the BCR. Conversely, co-ligation of the inhibitory co-receptor FcγRIIb with the BCR, leads to recruitment of SHIP to FcγRIIb and subsequent strong phosphorylation of SHIP by Lyn. As the PTB domain of Dok-3 is required for its binding to SHIP, strong phosphorylation of SHIP could lead to recruitment of Dok-3 to SHIP in the proximity of Lyn, leading to efficient phosphorylation.

To test this hypothesis, B cell lines of murine origin were used, since no gene homologous to *fcgr2b* is known in chicken and thus in the DT40 cell line. First, to test whether Grb2 is also required for Dok-3 phosphorylation in B cells of murine origin, Bal17.TR cells were used. This cells line is derived from Bal17 cells, which were subcloned and screened for a Grb2-deficient clone (Harmer and DeFranco, 1999). Re-expression of Grb2 in this clone indeed enhances Dok-3 tyrosine phosphorylation in response to BCR ligation, indicating an evolutionary conserved dependence on Grb2 expression (Figure 11, compare lanes 2 and 4). To test the effect of FcγRIIb on Dok-3 phosphorylation, FcγRIIb was co-crosslinked to the BCR by using complete rabbit IgG raised against the BCR. Note, that for stimulation of the BCR only, $F(ab)_2$ fragments of the same antibody were used. The phosphorylation of Dok-3 is drastically increased by co-ligating FcγRIIb to the BCR (Figure 11, lanes 6 and 7).

The effect of FcγRIIb co-ligation with the BCR on Dok-3 phosphorylation was confirmed using another mouse B cell line, WEHI-231 (Figure 12A, lanes 4-6). Interestingly, FcγRIIb co-ligation has previously been shown to induce phosphorylation of SHC and the Dok-family member Dok-1 and their binding to SHIP (Ott et al., 2002; Tridandapani et al., 1997). The enhanced phosphorylation of the former adaptor proteins could be recapitulated in WEHI-231 cells. The association of Dok-3 and SHC with SHIP was hardly altered in response to FcγRIIb co-ligation in WEHI231 cells

(Figure 12A, second panel). In immunoprecipitates of Dok-1, SHIP could not be detected, though, which might just be due to the poor quality of the anti-Dok-1 antibody.

To asses, whether SHIP forms a single complex with all three adaptor proteins or three individual complexes the immunoprecipitates of the adaptor proteins were also probed with antibodies to the respective others (Figure 12A, lower panels). Due to the reactivity of the secondary antibodies with the rabbit antibodies used for immunoprecipitating SHC and for co-ligating FcγRIIb this could not be shown, though. Only in Dok-3 immunoprecipitates, Dok-1 could be detected. As Dok-1 immunoprecipitation was inefficient with the used antibody, this could not be confirmed by detecting Dok-3 in anti-Dok-1 immunoprecipitates, though. This leaves the composition of the SHIP containing complexes undefined.

To asses that the effect on Dok-3 phosphorylation is indeed due to co-ligation of FcγRIIb by the whole anti-IgM antibody, FcγRIIb was blocked by using an anti-FcgRII antibody. As shown in Figure 12B, preincubation of WEHI-231 B cells with FcγRII blocking antibody can reverse anti-IgM induced phosphorylation of Dok-3.

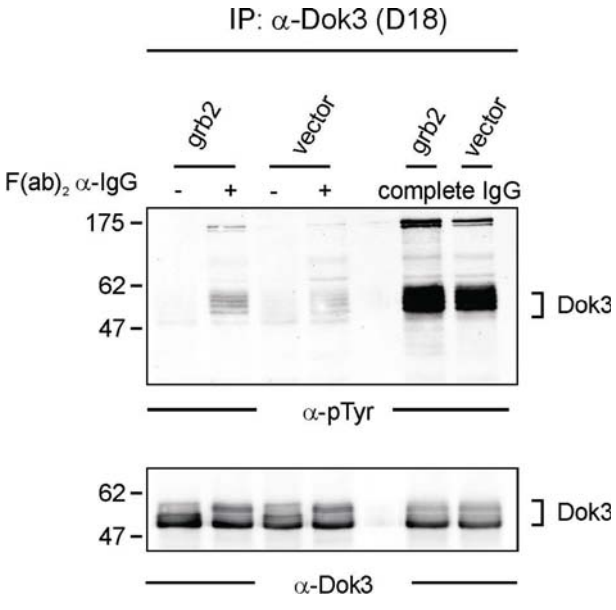

Figure 11 Dok-3 phosphorylation in mouse B cells is enhanced by Grb2 expression and FcγRIIb co-crosslinking
The Grb2-deficient mouse B cell line Bal17.TR, retrovirally reconstituted with wild-type Grb2 (lanes 1-2,6) or vector (lanes 3-4,7), was left untreated (0), stimulated through their BCRs using F(ab)2 fragments (+), or stimulated with co-ligation of FcgRIIb using whole IgG antibodies.. Lysates were subjected to anti-Dok-3 immunopurification and proteins obtained were analyzed by anti-pTyr and anti-Dok-3 immunoblotting (upper and lower panels, respectively).

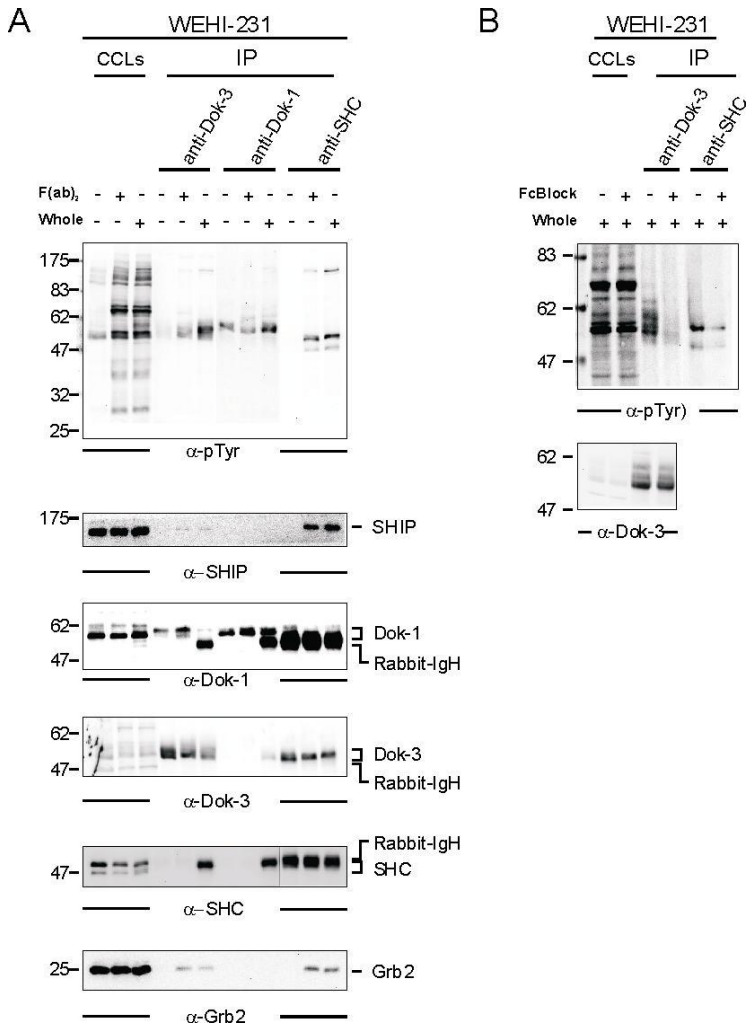

Figure 12:.Dok-3 phosphorylation is enhanced by FcgRIIb co-ligation in the B cell line WEHI-231.

(A) WEHI-231 cells were left untreated (-), stimulated through their BCRs using $F(ab)_2$ fragments, or stimulated with co-ligation of FcγRIIb using whole IgG antibodies, as indicated. Cleared cellular lysates (CCLs) were subjected to anti-Dok-3, anti-Dok-1 or anti-SHC immunoprecipitation and proteins obtained were analyzed by anti-pTyr, anti SHIP, anti-Dok-1, anti-Dok-3, anti-SHC and anti-Grb2 immunoblotting (from top to bottom). As whole IgG for coligation of BCR and FcgRIIb and the SHC antibody were derived from rabbit, crossreaction is observed in immunoblots against Dok-1, Dok-3 and SHC.

(B) WEHI-231 cells were left untreated (-) or preincubated with a blocking anti-mouse CD32 antibody and subsequently stimulated by anti-BCR whole IgG antibody, to co-crosslink BCR and FcγRIIb .CCLs were subjected to anti-Dok-3 or anti-SHC immunoprecipitation and proteins obtained were analyzed by anti-pTyr and anti-Dok-3 immunoblotting.

5.6 Dok-3 binds to phospho-tyrosine 1020 in SHIP

As shown in Figure 7, a Dok-3 mutant harboring an inactivated PTB domain does not associate with SHIP. This suggests that the Dok-3 PTB domain binds to a phosphorylated tyrosine motif in SHIP. Two PTB domain-binding motifs in SHIP, carrying the consensus binding sequence N-P-x-Y (x stands for any amino acid) have been described and both have been shown to bind the PTB domain of SHC (Lamkin et al., 1997). To elucidate, if Dok-3 binds to the very same motifs, SHIP mutants were generated. The mutants of the PTB binding sites ($Y^{917}F$ and $Y^{1020}F$) were expressed in SHIP-deficient DT40 cells, which had been transfected with the cDNA of human FcγRIIb, as DT40 cells do not seem to express an Fc receptor. Upon stimulation via the BCR alone or together with FcγRIIb, the ability of Dok-3 and SHC to associate with SHIP was assessed by co-immunoprecipitation. As shown in Figure 14A, wild type SHIP was co-immunoprecipitated with Dok-3. The interaction was increased by stimulation by the BCR and even stronger after FcγRIIb co-ligation. Mutation of Y917 led to a slightly reduced association of SHIP with Dok-3, but mutation of Y1020 completely abolished binding to Dok-3. This indicates that Dok-3 exclusively binds to the motif containing phospho-Y1020 in SHIP. Contrary to Dok-3, SHC binding to SHIP was only abolished, if both tyrosine motifs are mutated (Figure 13B). Interestingly, binding of both Dok-3 and SHC to Grb2 is augmented in this cell system upon FcγRIIb co-crosslinking. This seems to correlate with the former´s ability to bind to SHIP, indicating that Dok-3 may form a similar ternary complex with Grb2 and SHIP, as previously described for SHC (Harmer and DeFranco, 1999).

To test, if the interaction of Dok-3 with Y1020 of SHIP is mediated by its PTB domain, the recombinant PTB domain of Dok-3 was used to affinity purify SHIP from the same cells as used in Figure 14. To achieve full phosphorylation of the tyrosines in SHIP, cells were treated with the phosphatase inhibitor pervanadate. As shown in Figure 14, the PTB domain co-purified SHIP from pervanadate treated cells, while the same PTB domain rendered inactive by point mutation ($R^{297}A$) did not, showing that the PTB domain is sufficient for interaction. The intact PTB domain could co-purify SHIP harboring a mutation at Y917 but not at Y1020, demonstrating the interaction to occur at this tyrosine motif. Interestingly, a mutant of SHIP harboring a deletion in the

catalytic center could not be co-purified, although Y1020 is strongly phosphorylated. This mutant did also not show enhanced interaction with Dok-3 in cells stimulated by co-ligation of FcγRIIb as shown in Figure 13. This implicates that the deletion alters the

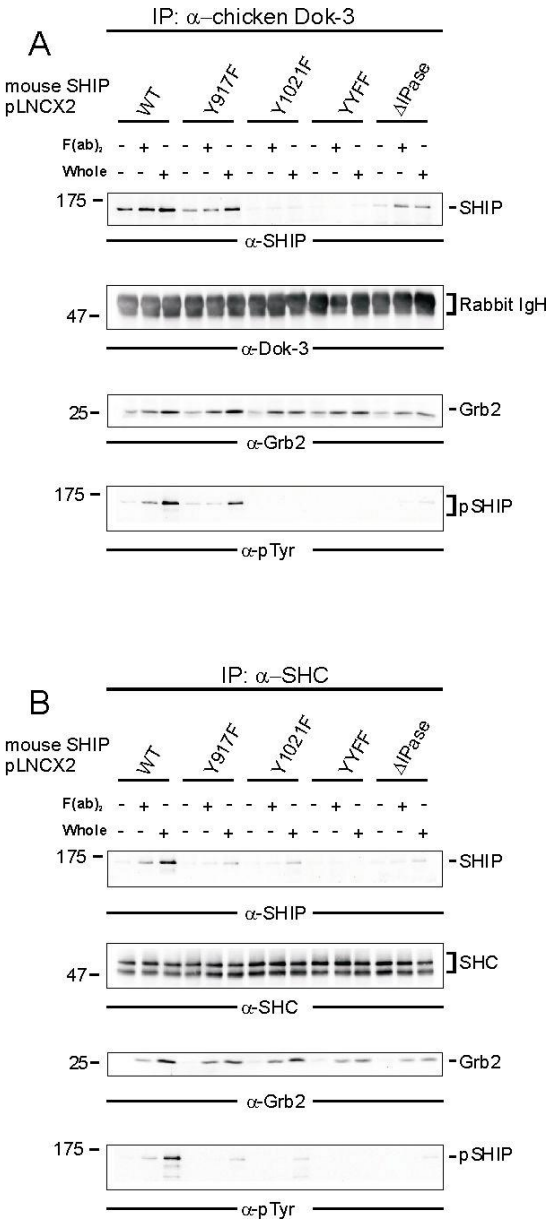

Figure 13: Y1020 in SHIP is essential for Dok-3 binding.
(A) SHIP-deficient DT40 cells, stably expressing human FcgRIIb, were retrovirally reconstituted with wild-type SHIP or mutants carrying Y to F substitutions at Y917, Y1020 or both (YYFF) and a mutant carrying an eight amino acid deletion in the catalytic domain (ΔIPase). Similar expression of the mutants was confirmed by Western blot analysis (data not shown). Variant cells were left untreated (-), stimulated through their BCRs by M4, or stimulated with co-ligation of FcγRIIb by addition of anti-M4 rabbit IgGs, as indicated. Cleared cellular lysates (CCLs) were subjected to anti-Dok-3 immunoprecipitation and proteins obtained were analyzed by anti-SHIP, anti-Dok-3, anti-Grb2 and anti-pTyr immunoblotting (from top to bottom).
(B) Lysates described in (A) were subjected to anti-SHC immunoprecipitation and proteins obtained were analyzed by anti-SHIP, anti-SHC, anti-Grb2 and anti-pTyr immunoblotting (from top to bottom).

conformation of SHIP as such that it does not bind to Dok-3 anymore. Binding to SHC is still inducible, probably via Y917. Note, that co-purification of the SHIP mutants is done using cell lysates containing native protein, while the phosphospecific antibody, recognizing Y1020 was used in Western blot analysis and thus using denatured protein.

Together, these data show that only phospho-Y1020 of SHIP is required for Dok-3 binding, while SHC can bind to both phospho-Y917 and phospho-Y1020. The PTB domains of SHC and Dok-3 thus seem to have partially overlapping binding sites. This implicates that they could either compete for binding or cooperate in binding as SHC could still bind to Y917. As co-immunoprecipitation of Dok-3 and SHC did not succeed, the phosphorylation of SHC in Dok-3-deficient cells was analyzed. The binding of SHC to SHIP is mandatory for its phosphorylation upon BCR stimulation (Ingham et al., 1999). As shown in Figure 15, phosphorylation of SHC is enhanced in the absence of Dok-3 expression. This argues rather for competition between Dok-3 and SHC to bind to SHIP and to be recruited to SHIP-bound Lyn. Further, Grb2 expression

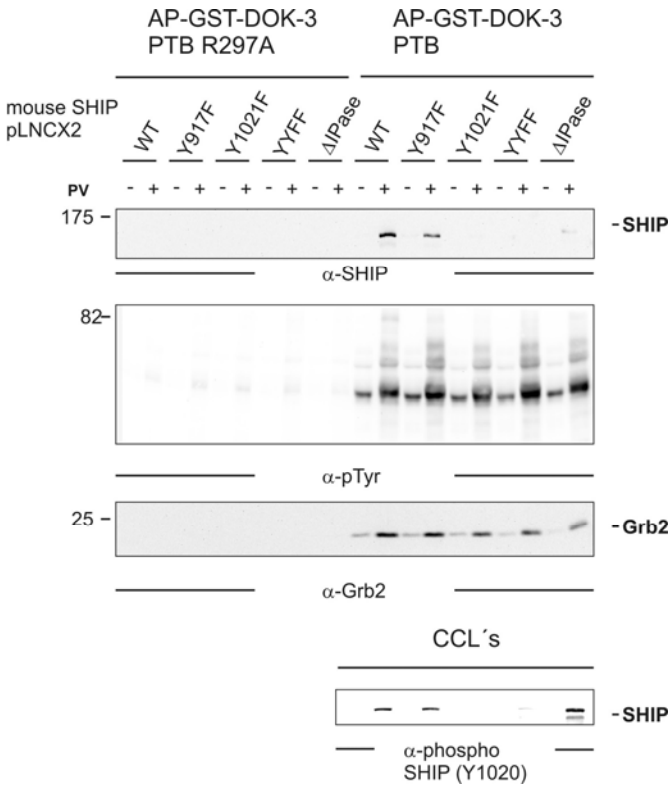

Figure 14:PTB domain of Dok-3 binds exclusively to Y1020 of SHIP.
Variants of SHIP-deficient DT40 cells, as described in Figure 13, were left untreated or were treated with pervanadate (+) to obtain maximum phosphorylation. Lysates were subjected to affinity purification using immobilized, recombinant PTB domain of Dok-3 (GST-Dok-3 PTB) or a phosphotyrosine binding inactive mutant (GST-Dok-3 PTB R297A). Affinity purified proteins were analyzed by anti-SHIP, anti-pTyr and anti-Grb2 immunoblotting. Efficient pervanadate induced phosphorylation of SHIP was confirmed by analyzing CCLs by anti phospho-SHIP immunoblotting.

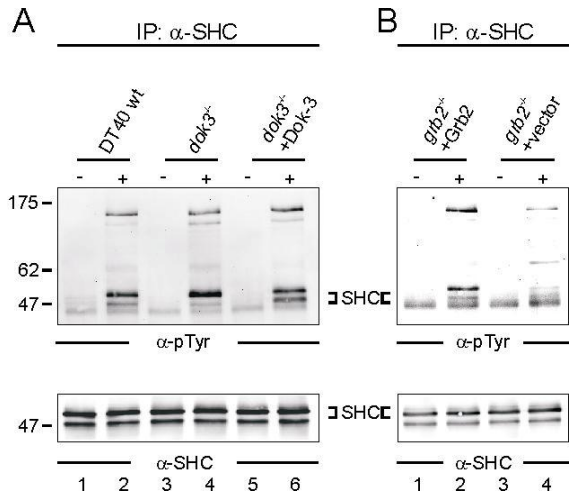

Figure 15: Increased SHC phosphorylation in Dok-3-deficient cells.
(A) *Wild-type DT40 cells (lanes 1-2), dok-3-/- mutants (lanes 3-4) and their reconstituted transfectants expressing HA-tagged Dok-3 (lanes 5-6) were left untreated (0) or stimulated through their BCR for 3min (+). Cleared cellular lysates (CCLs) were subjected to anti-SHC immunoprecipitation and proteins obtained were analyzed by anti-pTyr and anti-SHC immunoblotting (top block and lower block,respectively).*
(B) *Wild-type DT40 cells and mutants deficient for Grb2 were treated as described in (A)*

is required for SHC phosphorylation (Figure 15 B). As both Dok-3 and SHC are phosphorylated only in the presence of Grb2, they might not only compete for SHIP but also Grb2 binding. The complex interplay between SHIP, Grb2, SHC and Dok-3 has thus to be investigated in more detail.

6 Discussion

B lymphocyte development, survival and activation rely on signaling via the BCR. The outcome of signaling is regulated by constitutive or inducible positive and negative signaling elements. In this thesis one negative signaling module comprised of Grb2 and Dok-3 was shown to act constitutively and also inducibly after FcγRIIb co-crosslinking. FcγRIIb signaling has been shown to be critical for the establishment of peripheral tolerance (Takai et al., 1996). Apart from the recruitment and activation of the lipid phosphatase SHIP, downstream signaling events are only weakly characterized. The question remains, whether or how this receptor affects other negative or positive signaling modules.

6.1 The Dok-3/Grb2 module inhibits IP3-mediated ER Ca^{2+} store depletion

Crosslinking of the BCR by multivalent or membrane-bound antigens leads to a transient or sustained rise in cytosolic Ca^{2+} concentration. BCR-induced Ca^{2+} mobilization occurs in two phases. In the first phase, the binding of IP3 to its receptors in the ER membrane results in release of Ca^{2+} from ER stores. The depletion of the ER Ca^{2+} stores leads to the second phase of sustained influx of extracellular Ca^{2+} through plasma membrane channels.

The rise of the cytosolic Ca^{2+} concentration in BCR-stimulated cells can mainly be inhibited by three different mechanisms: First, inhibition of Ca^{2+} release from the ER, which also reduces subsequent store-operated Ca^{2+} entry. Second, the extrusion of Ca^{2+} from the cytosol to the extracellular space, which results in reduced cytoplasmic Ca^{2+} concentrations in both phases. Third, the inhibition of Ca^{2+} entry through plasma membrane Ca^{2+} channels.

During my work Björn Stork could show that the adaptor protein Dok-3 inhibits the rise in cytosolic Ca^{2+} concentration in both phases in response to BCR stimulation employing *dok-3*$^{-/-}$ DT40 B cells (Stork, 2006). However, for unknown reasons, overexpression of mouse Dok-3 does not inhibit Ca^{2+} mobilization (Robson et al.,

2004). Grb2 has previously been shown to inhibit Ca^{2+} mobilization in response to BCR ligation. As Grb2-deficient DT40 cells show especially stronger Ca^{2+} entry than their wild-type counterparts, the authors suggested a role in regulating Ca^{2+} entry across the plasma membrane (Stork et al., 2004).

This thesis now clearly establishes that both Grb2 and Dok-3 solely act on the first mechanism, inhibition of Ca^{2+} release from the ER Ca^{2+} stores. First, the amount of Ca^{2+} remaining in the ER after the signal terminated was significantly decreased in DT40 cells deficient for Grb2 or Dok-3 as compared to their wild type counterparts. *Per se*, this stronger degree of store depletion leads to increased store-operated Ca^{2+} entry (Engelke et al., 2007). If the efflux of Ca^{2+} from the cytoplasm to the extracellular space was compromised in the mutant cell lines, similar or even greater amounts of Ca^{2+} would have been expected to remain in the ER Ca^{2+} stores as compared to wild type counterparts. Second, the Ca^{2+} entry is not influenced by Grb2 or Dok-3 expression, when the ER is artificially depleted by the pharmacological agent Thapsigargin. Thus, CRAC channels seem not to be directly influenced by Dok-3 and Grb2. Very similar experiments have previously been described for SHIP-deficient cells, establishing SHIP´s role in inhibiting release of Ca^{2+} from internal stores only (Hashimoto et al., 1999).

6.2 Grb2 and Dok-3 inhibit Btk-mediated PLC-γ2 activation

As the release of Ca^{2+} from the ER is mediated by IP3 binding to its receptors, it was consequential to assay IP3 levels in both activated Grb2 and Dok-3-deficient cells. In both mutant cell lines the BCR-induced rise in IP3 levels was much stronger than in their wild-type counterparts. This could either be due to higher activity of PLC-γ2 or reduced IP3 degradation. The actual activity of PLC-γ2 was not tested in vitro, but phosphorylation at a tyrosine critical for activation of PLC-γ2 is strongly increased in Grb2 and Dok-3-deficient DT40 cells. This Y759 (of human PLC-γ2) is located in the linker region of PLC-γ2 and is phosphorylated by Btk after BCR stimulation. Phosphorylation by Btk at Y759 and Y753 has been shown to correlate with PLC-γ2 activation (Humphries et al., 2004; Kim et al., 2004)

In Grb2-deficient cells, phosphorylation of PLC-γ2 at Y759 could not be assayed due to a low signal to noise ratio and total tyrosine phosphorylation of PLC-γ2 was not changed as detected by a general anti-phosphotyrosine antibody. To circumvent this partly contradicting finding, Btk was overexpressed in Grb2-deficient cells. This consequently increased total tyrosine phosphorylation of PLC-γ2 and also phosphorylation at Y759. In cells overexpressing Btk, the influence of Grb2 on PLC-γ2 phosphorylation could be clearly shown. Not only phosphorylation on Y759, but even total tyrosine phosphorylation of PLC-γ2 is inhibited by Grb2 in cells overexpressing Btk. This shows on the one hand, that Btk-mediated phosphorylation of PLC-γ2 is

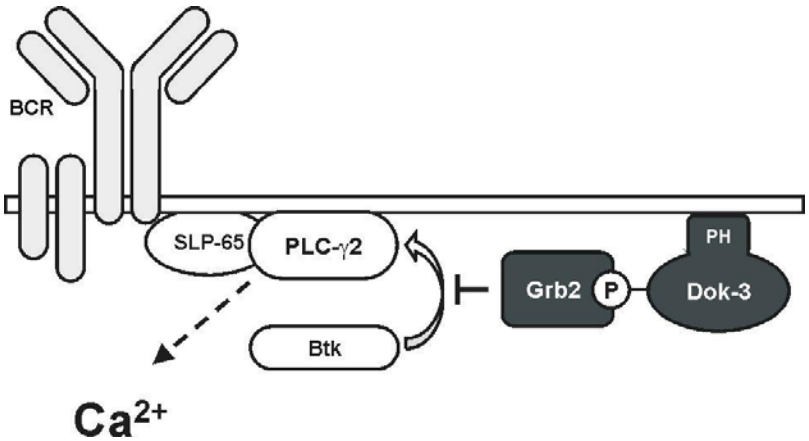

Figure 16: Inhibition of BCR-induced Ca2+ signaling by the Dok-3/Grb2 module. The Dok-3 adaptor protein is tehthered at the inner side of the plasma membrane by virtue of its PH domain. BCR-stimulation induces phosphorylation of Dok-3 and interaction with Grb2. This leads to reduced Btk-mediated activation of PLC-γ2 by interfering with the proper formation of the SLP-65-assembled Ca^{2+} initiatiation complex and/or inhibiting the enzymatic activity of Btk. Positive and negative regulators of Ca^{2+} mobilization are illustrated by open and filled boxes, resectively. The BCR complex is depicted in grey.

inhibited by Grb2. On the other hand, Btk-independent phosphorylation at other sites of PLC-γ2 seems not to be influenced by Grb2.

The total tyrosine phosphorylation of PLC-γ2 in response to BCR ligation is increased in Dok-3-deficient DT40 cells, which is not the case in Grb2-deficient DT40 cells. Also, IP3 production and Ca^{2+} mobilization is stronger in Dok-3-deficient DT40 cells, as compared to Grb2-deficient DT40 cells. Thus, the inhibitory function of Dok-3 seems to be stronger than that of Grb2. This notion is surprising as Grb2 is also required for efficient phosphorylation of SHIP, a well characterized inhibitor of Ca^{2+} mobilization in B cells. This discrepancy is either due to residual Dok-3 activity in the absence of Grb2 or more likely, additional positive regulatory functions of Grb2 in BCR signaling.

6.3 Dok-3 acts independently of SHIP

Data presented in this thesis show that both Dok-3 and Grb2 associate with SHIP in DT40 B cells stimulated via the BCR (Figure 7), which has been shown in mouse B cells before (Harmer and DeFranco, 1999; Lemay et al., 2000). Surprisingly though, data presented in this thesis implicate Dok-3 to act independently of SHIP. First, a SHIP binding-deficient mutant of Dok-3, in which the PTB domain was inactivated, showed similar inhibition of Ca^{2+}-flux as compared to wild-type Dok-3. Second, a mutant version of Dok-3, in which the binding site of Grb2 was mutated, acted as a dominant-negative mutant in both wild-type and SHIP-deficient DT40 cells. Third, phosphorylation of SHIP was only slightly reduced in Dok-3-deficient DT40 cells. And last, phosphorylation of Akt, which is inhibited by SHIP (Aman et al., 1998), was not altered in Dok-3-deficient DT40 cells.

This does not mean that the interaction between Dok-3 and SHIP is functionally irrelevant. In mouse B cells, showing less Dok-3 phosphorylation upon BCR stimulation, Dok-3 phosphorylation is enhanced by FcγRIIb co-ligation. This is most likely due to PTB domain-mediated recruitment of Dok-3 to phosphorylated SHIP. SHIP is recruited directly via its SH2 domain to FcγRIIb and co-localizes with Lyn under negative signaling conditions (Bewarder et al., 1996; Tridandapani et al., 1997), and thus could recruit Dok-3 into the proximity of Lyn, supporting its phosphorylation.

Thus, there seem to be at least 2 ways to recruit Dok-3 into the proximity of Lyn at the plasma membrane. In DT40 cells, a SHIP-independent mechanism leads to strong Dok-3 phosphorylation. In cells originating from other developmental stages or species, Dok-3 activation might require the second pathway via SHIP. Interestingly, Dok-3 is constitutively associated with the plasma membrane in DT40 cells (Goldbeck, 2007). In mouse fibroblasts Dok-3 is localized in the cytoplasm (Cong et al., 1999). The constitutive membrane anchoring of Dok-3 in DT40 cells might make SHIP-dependent membrane recruitment unnecessary. Further work will elucidate the mechanism of Dok-3 membrane/lipid raft recruitment in B cells of different species or developmental stages.

A very recent publication supports a function of Dok-3 in primary mouse B cells similar to that observed in DT40 B cells (Ng et al., 2007). B cells isolated from *dok3*$^{-/-}$ mice showed similar hyper responsiveness to BCR stimulation as the *dok3*$^{-/-}$ DT40 cells analyzed in this thesis. Interestingly, in B cells of the mutant mice, SHIP phosphorylation at Y1020 is severely compromised as compared to wild-type cells. This is contradictory to the data provided in this thesis and may emphasize the above notion that activation and maybe also function of Dok-3 is different in cells of different species and developmental stage. Still, the data presented in this thesis strongly suggest an additional mode of function of Dok-3 independent of SHIP.

The DT40 system employed in this thesis is not *per se* less meaningful as compared to gene-targeted mice. DT40 is a transformed cell line that it is less phylogenetically related to the human system than are mice. The DT40 system is advantageous in 2 respects: First, conventional gene targeting strategies in mice can lead to developmental differences, especially in B and T cells. Lymphocyte development depends on signals emanating from their immunoreceptors (preBCR/preTCR for development and BCR/TCR for positive and negative selection). Thus, differences in signal transduction of mature lymphocytes can just arise from distinct selection of progenitor cells. Second, reconstitution experiments proving the specificity of the results are harder to perform in primary cells. Thus, the DT40 system is powerful complementary system.

In contrast to Dok-3, Grb2 seems to have a stronger impact on SHIP phosphorylation and function (Figure 9), which has not been investigated in mice, as *grb2*$^{-/-}$ mice are not

viable (Cheng et al., 1998). Although Grb2 is required for Dok-3 function, as shown in this and previous work (Goldbeck, 2007; Stork, 2006), it is likely that Grb2 has further effects on BCR signaling. First, Grb2 is not only required for BCR induced phosphorylation of Dok-3, but also of SHIP and SHC (this work and (Harmer and DeFranco, 1999)). This could mean that Grb2 might have a more general role in recruiting target proteins to Lyn. Second, Grb2 has been shown to interact with a plethora of proteins involved in BCR signaling, e.g. SLP-65, CD2AP, HPK-1, Cbl, SHP-1 and SHP-2 just to name a few. The functional spectrum of Grb2 could even broaden, as it has been shown to be phosphorylated at two tyrosine residues in its C-terminal SH3 domain downstream of the of the epidermal growth factor receptor (Olsen et al., 2006). Last, the phenotype of Grb2-deficient DT40 cells is less severe in terms of enhanced Ca^{2+}-flux and IP3 production as compared to Dok-3 or SHIP-deficient cells. This argues for additional positive roles of Grb2 in BCR signaling, which are absent in *grb2*$^{-/-}$ cells as well. Interestingly, such a role has been described in other receptor systems. Recently, Grb2 has been shown to be essential for recruitment of the p85 subunit of PI3K to DAP10 and subsequent Ca^{2+} flux (Upshaw et al., 2006). This NKG2D-associated co-receptor has a similar Grb2-binding site as the co stimulatory co-receptor of T cells, CD28, which also binds to Grb2 (Kim et al., 1998). Also, binding to LAT or the closely related NTAL has been shown to positively regulate signaling (Houtman et al., 2006; Stork et al., 2004). These data strongly suggest a pleiotropic effect of Grb2 also in B cell signaling with the cellular context determining the net outcome, positive or negative.

6.4 SHIP-SHC-Dok complexes

The interaction of SHIP with SHC has been investigated in detail (Harmer and DeFranco, 1999). SHC binds via its PTB domain to phosphorylated Y917 or Y1020 of SHIP. This interaction in not sufficient to induce stable complex formation, though. Grb2 stabilizes the interaction by binding to both SHC and SHIP, which leads to formation of a ternary complex. Grb2 binds to a phosphorylated tyrosine in SHC via its SH2 domain and to SHIP via its C-terminal SH3 domain (Figure 17A). Similar to SHC, also Dok-3 binds via its PTB domain to SHIP, albeit exclusively to Y1020. Further, Grb2 binds to Dok-3 Y331 via its SH2 domain and could thus also stabilize the Dok-

3/SHIP interaction by simultaneously binding to SHIP via its SH3 domain (Figure 17B). Harmer et al. showed that supplementing lysates of Grb2-deficient B cells with recombinant Grb2 enhances SHC/SHIP interaction, proving that Grb2 binds to both proteins simultaneously. This kind of experiment is planned for the Dok-3/SHIP interaction, and will show whether the herein proposed binding model is true.

As Dok-3 and SHC seem to bind to SHIP in a similar fashion, it is feasible to propose a model in which SHIP can form two distinct ternary complexes, either with Grb2 and SHC or with Grb2 and Dok-3, which is coined herein as the "Alternative Binding Model". This model would exclude a simultaneous binding of SHIP to both SHC and Dok-3, and thus a complex containing both SHC and Dok-3. Co-immunoprecipitation studies during this thesis could not show an interaction of SHC and Dok-3, but a negative result is of course not a proof, that this interaction does not occur. To prove the model, it has to be shown, that SHC and Dok-3 compete for binding to SHIP, rather than cooperate in binding to SHIP. This is supported by the fact, that SHC phosphorylation, which requires binding to SHIP (Ingham et al., 1999), is increased in Dok-3-deficient DT40 cells, albeit mainly the longer isoform (p52) (Figure 15). To show competitive binding, recombinant SHC or Dok-3 could be added to lysates of stimulated B cells. According to the model, the association of the respective other protein with SHIP should then decrease. Using the two isoforms of SHC could then also elucidate a potential distinct mode of action of those. As phosphorylated recombinant

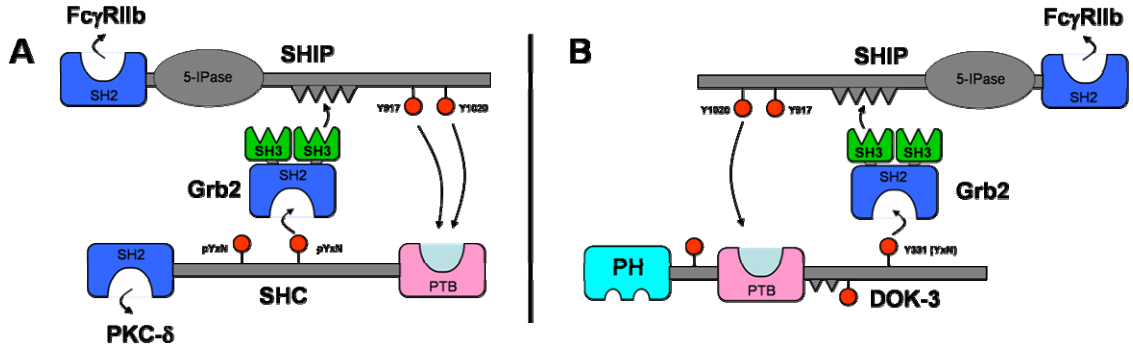

Figure 17: Alternative binding model. A) SHC binds to SHIP Y917 or Y1020 via its PTB domain and Grb2 stabilizes the interaction by binding to SHC via its SH2 domain and to SHIP via its C-terminal SH3 domain; adapted from (Harmer and DeFranco, 1999). B) Dok-3 binds to SHIP Y1020 via its PTB domain. Grb2 binds to Dok-3 Y331 via its SH2 domain and presumably at the same time to SHIP via its C-terminal SH3 domain, which leads to a ternary comlex, similar to complex formed by SHC.

SHC or Dok-3 is required, purification strategies for tagged proteins from DT40 are currently being established.

6.5 Role of Dok-3 in FcγRIIb signaling

The data provided in this thesis indicate that SHIP associates with Dok-3 after FcγRIIb coligation and previous publications indicate that SHIP associates also with Grb2, SHC, and Dok-1 after FcγRIIb coligation (Harmer and DeFranco, 1999; Ott et al., 2002; Tamir et al., 2000). Except for Dok-1, which inhibits ERK activation, no functional significance of any of these interactions for FcγRIIb signaling has been shown. In murine FcγRIIb, a Grb2-binding site is present, which enhances SHIP recruitment, but this site is not conserved in the human orthologue (Isnardi et al., 2004). Binding of SHC and Grb2 to SHIP has been suggested to inhibit Ras activation by sequestering the former from SOS, but SOS has later been shown to be dispensable for Ras activation in DT40 B cells (Oh-hora et al., 2003; Tridandapani et al., 1998). In the DT40 system Grb2, Dok-3 and SHC are not essential for inhibition of Ca^{2+} mobilization by FcγRIIb (data not shown). As Dok-3 is clearly a strong inhibitor of Ca^{2+} mobilization in response to BCR ligation, this seems surprising at first. This can be explained by the already strong phosphorylation of Dok-3 after BCR crosslinking only, which is independent of SHIP expression (B.Stork, unpublished observation). Co-ligation of FcγRIIb consequently does not enhance Dok-3 phosphorylation in this system (data not shown). Note that DT40 cells seem not to express an Fc receptor similar to FcγRIIb. This implies that DT40 cells or chicken B cells in general have a distinct mode of Dok-3 activation, making this system unfavorable for investigating its role in FcγRIIb signaling. Further, Dok-3 acts on Btk-mediated PLC-γ2 activation, which is the very same mechanism by which SHIP inhibits Ca^{2+} mobilization (Bolland et al., 1998). This makes it difficult to distinguish between SHIP and Dok-3 function.

Dok-3 phosphorylation depends on Grb2 expression, and in DT40 cells Dok-3 is the main Grb2-binding protein after BCR stimulation (Goldbeck, 2007). B cells from other developmental stages or species express other Grb2-binding proteins, like the transmembrane adaptor NTAL. In these cells, Dok-3 has to compete for binding to Grb2, which may be one reason for low Dok-3 phosphorylation in mouse B cells. This

implies that high expression of NTAL in DT40 cells should lead to decreased BCR-mediated Dok-3 phosphorylation. It would be interesting to see, whether FcγRIIb co-ligation would lead to again increased Dok-3 phosphorylation.

Anyhow, to see whether the adapter proteins recruited to SHIP bring additional signaling functions or solely regulate SHIP activity remains to be elucidated. This was attempted during this thesis by expressing a catalytically inactive mutant of SHIP, but the deletion mutant shown in Figure 13 seems to have a distorted structure and a point mutation (D676G) resulted in strongly reduced expression compared to wild-type SHIP. Together, these drawbacks make the DT40 system unfavorable for the analysis of FcγRIIb signaling.

Immuncomplexes that do not contain antigen recognized by the BCR can crosslink FcγRIIb alone, which induces apotosis. The pathway leading to apoptosis is independent of the ITIM and SHIP, but requires activity of members of the Abl family of tyrosine kinases (Tzeng et al., 2005). Indeed, SHIP expression even inhibits apoptosis induced by FcγRIIb. This led the authors to propose, that the two pathways emanating from FcγRIIb block the respective other to fully skew a response to one pathway, either recruitment of SHIP or Abl. As Dok-3 inhibits Abl activity (Cong et al., 1999) it would be feasible to propose, that the Dok-3 function downstream of FcγRIIb could be to block the Abl dependent pathway to apoptosis.

6.6 Outlook

It is shown in this thesis that Dok-3 and Grb2 together inhibit Btk-mediated phosphorylation of PLC-γ2. As none of the known binding partners of Dok-3 seems to be involved in this and Grb2 is also an adaptor protein, another unknown protein seems to be required. Attempts to identify proteins interacting with Dok-3 in response to BCR stimulation have been undertaken. Tagged Dok-3 was purified from stimulated DT40 cells but no co-purified interacting partners could be identified yet. In a similar approach using a tagged version of SLP-65 several known and unknown binding partners of SLP-65 could be identified by mass spectrometry (K. Neumann, T. Oellerich, unpublished). Interestingly, also Dok-3 was co-purified with SLP-65. As background proteins were subtracted using the recently developed method of stable isotope labeling in cell culture (SILAC) (Ong et al., 2002), this strongly suggests Dok-3 to associate with SLP-65 and thus with the Ca^{2+} initiation complex. Further experiments will show whether the association is direct or via Grb2 and whether this interaction is able to perturb the integrity of the Ca^{2+} initiation complex, which would nicely explain the phenotype of the *dok3*$^{-/-}$ cells.

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8 Addendum

8.1 List of Figures:

8.2 List of tables:

Curriculum vitae

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since 2004/11	Scientific assistant in the Dept. of Cellular & Molecular
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Göttingen, 2008/11/25

Eidesstattliche Erklärung:

Hiermit erkläre ich an Eides Statt, die vorliegende Dissertation selbständig und ohne unzulässige Hilfe Dritter unter ausschließlicher Nutzung der aufgeführten Materialien, Methoden und Literaturquellen an der Universität Bielefeld und an der Georg-August-Universität Göttingen unter der Leitung von Prof. Dr. J. Wienands angefertigt zu haben.

Göttingen, den 25.11.2008 Konstantin Neumann

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