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The B-lymphoid Grb2 interaction code

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Summary: The growth factor receptor-bound protein 2 (Grb2) is a ubiquitously expressed and evolutionary conserved adapter protein possessing a plethora of described interaction partners for the regulation of signal transduction. In B lymphocytes, the Grb2-mediated scaffolding function controls the assembly and subcellular targeting of activating as well as inhibitory signalosomes in response to ligation of the antigen receptor. Also, integration of simultaneous signals from B-cell coreceptors that amplify or attenuate antigen receptor signal output relies on Grb2. Hence, Grb2 is an essential signal integrator. The key question remains, however, of how pathway specificity can be maintained during signal homeostasis critically required for the balance between immune cell activation and tolerance induction. Here, we summarize the molecular network of Grb2 in B cells and introduce a proteomic approach to elucidate the interactome of Grb2 *in vivo*.

Keywords: *B-cell activation, adapter proteins, SH2 and SH3 domains, protein tyrosine kinases, lipid phosphatases*

Introduction: a brief historical perspective on Grb2 structure and function

The discovery of adapter proteins and the elucidation of their role in signal transduction are intrinsically tied to the scientific history of oncogenes. In fact, a hallmark in the understanding of neoplastic cell transformation and human oncogenesis was the identification and molecular cloning of enzymatically inert adapter proteins as proximal effectors for cell surface receptors with intrinsic protein tyrosine kinase activity. Lowenstein *et al.* (1) used a biochemical approach to globally purify proteins that can bind to the tyrosine-phosphorylated forms of the epidermal growth factor receptor (EGFR) or the platelet-derived growth factor receptor. Several candidate proteins were identified and accordingly numbered. The growth factor receptor-bound protein 2 (Grb2) was found to be entirely composed of non-catalytic protein–protein interaction modules, which were previously recognized as evolutionary conserved building blocks of Src-family kinases and hence named Src homology (SH) domains (2). In a cDNA library screen for mammalian genes encoding SH2 domain-containing proteins, Matuoka *et al.* (3) independently identified Grb2 as a molecule

called Ash for 'abundant Src homology'. Grb2 encompasses one central SH2 domain flanked on either side by one SH3 domain. SH2 and SH3 domains can bind to short consensus peptide motifs which harbor a phosphorylated tyrosine residue or a proline-rich recognition site with a PXXP core, respectively (4). The cognate ligand can be recognized by the SH2 or the SH3 domain *in cis* as well as *in trans* allowing for intra- and intermolecular interactions.

The binding preferences of the Grb2 domains have been extensively studied in many cellular systems. The consensus motif for the Grb2 SH2 domain requires an asparagine (5) and prefers a methionine (6) in the phosphotyrosine +2 and +3 position, respectively. Moreover, under conditions where different SH2 domains compete for binding (as it is the case in the live cell), only the Grb2 SH2 domain tolerated a positively charged arginine residue in the +1 position of the negatively charged phosphotyrosine (6). Hence, a highly selective ligand for the Grb2 SH2 domain is pYRNM. The N-terminal SH3 domain has been shown to interact with a typical polyproline helix motif encompassing a PxxPxR sequence (7, 8), An atypical motif, PXXXR/KXXKP, lacking the classical PXXP core is bound with high affinity by the C-terminal SH3 domain of Grb2 with a remarkable versatility (9, 10). Many of the reported ligand proteins associate with Grb2 *in vivo* through a concerted action of the Grb2 domains in a constitutive and stimulation-dependent manner. The structure of full-length Grb2 was determined by X-ray crystallography at 3.1 Å (11) as well as in solution by nuclear magnetic resonance spectroscopy and small angle X-ray scattering measurements (12). The crystal structure shows a compactly folded protein with intramolecular contacts between the SH3 domains. In solution, Grb2 exhibits extensive flexibility facilitating ligand recognition.

The Grb2 architecture is highly conserved during evolution. Dependent on the species from which it is isolated, Grb2 migrates in conventional gel electrophoresis with an apparent molecular mass of 25–30 kDa. While Grb2 is ubiquitously expressed, the Grb2-related adapter protein (Grap), which shares almost 60% amino acid sequence identity to Grb2, is predominantly but not exclusively expressed in spleen and thymus (13). A hematopoietic cell type-specific expression pattern is reported for the third Grb2 family member, Grb2-related adapter downstream of Shc (Gads) (14). Gads is alternatively called GrpL (Grb2-related protein of the lymphoid system) (15), Mona (monocytic adapter)(16), GRID (Grb2-related protein with insert domain) (17), Grf40 (Grb2 family member of 40 kDa) (18), or Grap-2 (19).

An early key to the understanding of a major Grb2 function was its striking structural similarity to the product of the *Caenorhabditis elegans* gene Sem-5, which together with Let-23 (encoding an EGFR-like protein) and Let-60 (encoding a p21Ras-like protein) controls vulval induction during nematode development (1, 3, 20). Another line of evidence supporting that Grb2 is directly involved signal transduction from cell surface receptors to the Ras pathway came from studies on a homeotic gene in *Drosophila* called sevenless. Loss of sevenless function abrogated development of the R7 photoreceptor in the compound eye of the insect (21). Sevenless turned out to encode a transmembrane receptor with intrinsic tyrosine kinase activity (22), and its effector Drk (downstream of receptor kinase) was identified as the *Drosophila* homologue of Sem-5/Grb2 (23, 24). Landmark publications in 1993 (23–30) ultimately unraveled a basic mechanism of how receptor tyrosine kinases can trigger Ras activation via the conserved SH2/SH3 domain-containing linker Grb2/Sem-5/Drk. Ligand-induced crosslinking of the surface receptor stimulates trans-phosphorylation on tyrosine residues in the cytoplasmic receptor domains leading to the SH2 domain-mediated recruitment of cytosolic Grb2, which is itself constitutively complexed via its two SH3 domains to a guanine nucleotide exchange factor (GEF) called son of sevenless (SOS). The stimulation-dependent membrane translocation process targets SOS into the vicinity of its substrate p21Ras, which is tethered to the inner leaflet of the plasma membrane via post-translational lipid modifications (31). SOS-mediated exchange of GDP for GTP activates Ras. Notably, human Grb2 and *Drosophila* Drk can functionally replace Sem-5 signaling in *C. elegans* (32). A variation on this common theme is the additional inclusion of another SH2-containing adapter protein called SHC (33). Here, it is SHC that binds to the phosphorylated receptor tail through its N-terminally located SH2 domain and subsequently recruits the Grb2/SOS complex via phosphotyrosine residues located in the central region of SHC (34). A second phosphotyrosine-binding module (PTB) is found at the C-terminal end of SHC.

It appeared a matter of time until Grb2 together with or without the help of SHC would be identified as the linker module that couples the activated B-cell antigen receptor (BCR) to SOS and subsequently to Ras. However, the BCR does not fit into the Grb2/SOS paradigm. In fact, Grb2 was found to be dispensable for BCR-induced Ras activation but to exert multiple functions implicated in activating and inhibiting B-cell activation. A comprehensive understanding of the Grb2 network in B cells is still being sought. We describe in this review the major B-cell functions of Grb2 and introduce a

proteomic approach to elucidate *in vivo* interaction partners of Grb2.

Grb2 and SOS are dispensable for BCR-induced Ras activation

In contrast to cell surface receptors with intrinsic kinase activity, the BCR connects to cytoplasmic tyrosine kinases of the Src and Syk family through the immunoreceptor tyrosine-based activation motifs (ITAMs) in the BCR signaling subunits Ig-α and Ig-β (35, 36). Early studies revealed triggering of the Ras/Raf/MAPK pathway in response to BCR ligation (37). The likely possibility that this activation step involves the canonical Grb2/SOS module was initially supported by several observations. Crosslinking of the BCR induced tyrosine phosphorylation and membrane translocation of SHC (38–40), which can potentially interact with the phosphorylated BCR (41, 42) and is also found in complexes containing Grb2 and SOS (43–45). It thus came as a surprise that ablation of SOS expression by gene targeting in the DT40 B-cell line strongly compromised receptor tyrosine kinase- but not BCR-induced Ras activation (46). It is now clear that another GEF activity provided by the class of Ras guanosine nucleotide-releasing proteins (RasGRPs) (47) is predominantly responsible for Ras activation in B cells.

The BCR couples to RasGRPs via phospholipase C-γ2 (PLC-γ2). Activation of this enzyme is initiated by Syk bound via its tandem SH2 domains to a doubly phosphorylated ITAM. The proximal Syk substrate is the adapter SH2 domain-containing leukocyte protein of 65 kDa (SLP-65)/B-cell linker (BLNK) (48, 49). Phosphorylated SLP-65 recruits PLC-γ2 and its upstream regulator Bruton's tyrosine kinase (Btk) into a trimolecular complex allowing for PLC-γ2 activation and subsequent hydrolysis of membrane phospholipids into membrane-bound diacylglycerol (DAG) and soluble inositol-trisphosphate (IP3) (50, 51). The DAG product recruits RasGRP family members by virtue of their C1 domain (46, 52, 53). Full GEF activity of membrane-anchored RasGRPs is achieved upon phosphorylation on threonine residues, which requires membrane recruitment and activation of protein kinase C by DAG and IP3-triggered Ca^{2+} mobilization, respectively (54, 55).

B cells express RasGRP isoforms 1 and 3. RasGRP1 is recruited to the plasma membrane following BCR engagement (52, 56), and overexpression of RasGRP1 augments Ras signaling (52). However, BCR-induced Ras activation appears to be normal in RasGRP1-deficient DT40 B cells (46). By marked contrast, ablation of RasGRP3 expression almost abrogated BCR-regulated Ras signaling in DT40 cells (46) and in mice (57). Hence, the activated BCR employs the Syk/SLP-65/PLC-γ2 pathway for membrane translocation of RasGRP3 as the main GEF activity for Ras. As shown more recently, RasGRPs and SOS can cooperate in Ras activation, which may be particularly important to amplify Ras activation under weak stimulation conditions (58).

Grb2 can integrate inhibitory coreceptor signals into the BCR machinery

The SH2 domain-containing 5′-inositol phosphatase (SHIP) (59, 60) complexed with phosphorylated SHC was among the first *in vivo* ligands for Grb2 to be described in B cells (4, 39, 61–64). The interaction was mapped to the C-terminal SH3 domain of Grb2, which associates with one or more of the proline-rich consensus binding motifs present in the C-terminus of various SHIP isoforms. Importantly, co-clustering of the BCR with the negative regulatory Fc receptor for IgG (FcγRIIb) was required for both maximal tyrosine phosphorylation of SHIP and its association with SHC (65).

SHIP is the major effector of FcγRIIb-mediated signal inhibition that limits B-cell activation (66–69). Dephosphorylation of the 5′-position in phosphatidylinositides by activated SHIP targets membrane recruitment of signaling proteins with a pleckstrin homology (PH) domain (70) that posses a binding specificity for such phospholipid moieties. The most prominent example is inhibition of membrane translocation of the Tec family kinase Btk (71, 72). As a consequence, BCR-induced Ca^{2+} mobilization and B-cell function are dramatically reduced by simultaneous co-engagement of FcγRIIb (73–77). However, even in the absence of FcγRIIb interference, the BCR-induced Ca^{2+} response of SHIP-deficient B-cell mutants is greatly elevated compared with that of their wildtype counterparts (77). This observation suggests a BCR-autonomous linkage to SHIP as part of a feedback inhibition circuit. Moreover, other Ca^{2+}-mobilizing enzymes whose BCR-induced activation is sensitive to inhibition of PH domain-mediated membrane recruitment are potential SHIP targets. For example, SHIP has been reported to inhibit the PKB/Akt-triggered B-cell survival pathway (78), which is downstream of phosphatidylinositol 3-kinase (PI3K) and requires several PH domain/phospholipid interactions for activation (79, 80).

Although SHIP-mediated signal inhibition of B-cell activation is now well established, the exact role of the SHIP ligand Grb2 for this process is not entirely clear. Binding of SHIP to the phosphorylated immunoreceptor tyrosine-based inhibi-

tion motif (ITIM) (81) in the FcγRIIb cytoplasmic domain can occur directly via the SH2 domain of SHIP (66–69). However, a second tyrosine-based phosphorylation site in FcγRIIb is of the YXN type, and binding to the SH2 domain of either Grb2 or Grap was necessary for robust co-immunoprecipitation of SHIP isoforms with FcγRIIb (82). This finding suggests that stable anchoring of SHIP at the FcγRIIb tail requires the formation of a ternary complex in which SHIP binds directly to the phospho-ITIM and indirectly to the phospho-YXN motif via its linkage to the SH3 domain of either Grb2 or Grap. It is assumed that the subcellular relocalization process of SHIP rather than the regulation of its enzymatic activity is the major determinant for SHIP action on its substrate (83). Hence, Grb2 and Grap act as an amplifier of SHIP-mediated signal inhibition downstream of FcγRIIb.

Two other prominent ligand-regulated coreceptors, CD22 and CD72, downmodulate BCR-induced Ca^{2+} flux by virtue of their cytoplasmic ITIM sequences. Upon phosphorylation, CD22 and CD72 directly recruit the SH2 domain-containing protein tyrosine phosphatase SHP-1, resulting in dephosphorylation of proximal BCR effector proteins including Ig-α/β and SLP-65 (84, 85). In addition to the SHP-1 effector and in analogy to FcγRIIb, Grb2 is recruited via its SH2 domain to phospho-YXN motifs present in both CD22 (86, 87) and CD72 (88, 89). Moreover, the SHP-2 isoform and Grb2 can directly bind each other via the SH2 and C-terminal SH3 domains of Grb2, although these interactions have not been demonstrated to occur in B cells (90, 91).

In summary, negative regulatory phosphatases with specificity for either membrane phospholipids or tyrosine-phosphorylated proteins are powerful tools to inhibit BCR activation at an early step. The implementation of Grb2 into that process allows modulation of the binding avidity between the coreceptor and the phosphatase and connection of the phosphatases to those coreceptors that lack an appropriate ITIM sequence to directly recruit the enzyme via the SH2 domain. The latter option greatly expands the number of modulatory coreceptor species that are able to use SHIP, thereby increasing the possibilities to fine tune B-cell responses.

Subcellular navigation of Grb2 by transmembrane and membrane-associated adapters controls positive versus negative signal output

The ability of Grb2 to act as a negative regulatory element during BCR-induced Ca^{2+} flux was directly demonstrated by the analysis of Grb2-deficient DT40 B-cell mutants (92), which had been generated by gene targeting (93). Lack of Grb2 expression enhances the release of Ca^{2+} from intracellular stores of the endoplasmic reticulum as well as the subsequent entry of extracellular Ca^{2+} through plasma membrane channels. The sustained biphasic Ca^{2+} profile of the parental DT40 cells was restored by re-introduction of wildtype Grb2 but not by Grb2 mutants in which either the SH2 or C-terminal SH3 domain had been inactivated by point mutations. The partner molecule of Grb2 in this inhibitory cascade is the hematopoietic adapter protein downstream of kinase-3 (Dok-3) (94, 95), which upon phosphorylation by Lyn provides specific pYXN docking sites for the Grb2 SH2 domain (96). Dok-3 contains an N-terminal PH domain for lipid raft localization followed by a PTB domain that binds phosphorylated SHIP. Mutational analysis and reconstitution experiments in Dok-3-negative DT40 B cells revealed that loss of membrane association abrogates Dok-3 phosphorylation, Grb2 recruitment, and concomitant inhibition of Ca^{2+} flux. The cascade did surprisingly not require SHIP (96). When bound to Dok-3 in lipid rafts via the SH2 domain, Grb2 suppresses Btk-mediated PLC-γ2 activation either by allosteric inhibition of Btk activity or by preventing incorporation of Btk into the SLP-65-assembled Ca^{2+} initiation complex. Ablation of Dok-3 expression in mice did not affect B-cell development but resulted in hyperproliferation of the B cells and in enhanced activation of several BCR signaling cascades including Ca^{2+} mobilization (97).

Grb2 is produced in almost similar amounts during all stages of B-cell development. Yet, the shape of the Ca^{2+} mobilization profiles markedly differs between various B-cell subpopulations (50, 98). DT40 B cells represent an immature stage of development in which B cells exhibit a more moderate Ca^{2+} response. It is thus likely that mechanisms to subvert inhibitory Grb2 signaling in mature immunocompetent B cells exist. One identified antagonist of the Dok-3/Grb2 module is the non-T-cell activation linker (NTAL) (92). NTAL was identified based on its structural homology to linker for activation of T cells (LAT) and is therefore also called LAT2 (99). LAT and NTAL belong to the family of transmembrane adapter proteins characterized by a short extracellular peptide stretch, a transmembrane helix, and an intracellular segment accommodating a number of protein–protein interaction sites (99). Many of the family members, including LAT and NTAL, carry post-translational fatty acid modifications at conserved cysteine residues in the juxtamembrane part and are permanent residents of lipid rafts. Linker of activated X cells (LAX) (97), which is also present in B cells, resides outside of the lipid rafts. A common and functionally relevant feature of transmembrane adapters is their ability to associate with Grb2

via one or more pYXN sites. Recruitment of Grb2 to phosphorylated NTAL counteracts its capacity to attenuate BCR-induced Ca^{2+} fluxes (92). The mobilization profile of NTAL-positive B cells is almost identical to that of Grb2-negative cells. By introducing NTAL expression, the moderate Ca^{2+} flux of NTAL-negative DT40 B cells was converted into a robust response reminiscent to that of mature B cells. Expression analysis in primary mouse B cells and B-cell lines showed that NTAL expression increases as the cells mature.

The phosphorylated adapter proteins Dok-3 and NTAL provide two functionally inverse membrane anchors for cytosolic Grb2 in B cells. The available data suggest that NTAL sequesters Grb2 away from Dok-3, a liaison, which drags Grb2 into a microenvironment that allows for inhibition of the Ca^{2+} initiation complex. Hence, NTAL is not a *bona fide* positive B-cell regulator but acts more like a mute button for Dok-3 action. Functionally redundant molecules of NTAL are likely to exist, as indicated by the mild immunological phenotype of NTAL-negative mouse mutants (100). NTAL has been shown in other cell types, notably T and mast cells, to function as a negative regulator by counteracting LAT (99). LAT is the critical membrane-anchoring platform for the Ca^{2+} initiation complex in T cells and therefore associates with Gads and PLC-γ1. NTAL lacks the PLC-γ1-binding site but efficiently binds Gads. Thus, when simultaneously expressed, NTAL competes with LAT for Gads binding and reduces the number of available Gads molecules for the Ca^{2+} initiation complex. The principle mechanism is basically the same as for Dok-3 regulation in B cells, i.e., phosphorylated NTAL provides an alternative membrane destination for a Grb2 family member. Whether the net result of this alternative binding is stimulatory or inhibitory for the cell is determined by the function of the Grb2 binder that is counteracted by NTAL. It also is a main and more general lesson learned from these studies that signaling controlled by the subcellular relocalization of Grb2 not just involves transportation of the protein from the cytosol to the plasma membrane. Rather, precise targeting to individual lipid raft residents is a key parameter of signal output. This suggests that even within the lipid rafts, the lateral mobility of proteins is limited, which prevents their random interaction and unwanted crosstalk between signal pathways.

Amplification of B-cell activation by incorporation of Grb2 into the BCR and the CD19 coreceptor

No direct association between the BCR and Grb2 had been reported until recently. Consistent with a lack of a BCR/Grb2 interaction is the absence of consensus phosphorylation motifs of the YXN type and appropriate proline-rich recognition motifs that might provide Grb2 docking sites in Ig-α and Ig-β. However, the cytoplasmic segments of membrane-bound IgG and IgE (mIgG and mIgE, respectively) contain an evolutionarily conserved tyrosine residue followed by an asparagine in the +2 position. In mIgG isoforms, the Y + 1 position carries a positively charged arginine (except for murine γ1, which harbors a lysine), and a methionine is present in the Y + 3 position. In the case of the mIgE cytoplasmic segments, the Y + 1 and Y + 3 positions vary between different species. Our group showed that the conserved tyrosine residue, now named as immunoglobulin tail tyrosine (ITT), underwent inducible phosphorylation and Grb2 recruitment following stimulation of the IgG- and IgE-BCR (101). ITT/Grb2 complex formation boosted Ca^{2+} mobilization and proliferative responses in various B-cell lines and primary B cells of the mouse. Mutant BCRs in which the ITT was inactivated by a phenylalanine substitution showed almost identical responses to tailless mIg variants or to the IgM-BCR. Hence, the ITT is a key element that distinguishes IgG/E-BCR signaling on class-switched memory B cells from that of the IgM-BCR expressed on newly generated cells. The data explain the observed burst-enhancing role of the IgG tail segment in genetically engineered mouse models (102–105).

Several mechanisms for how an ITT/Grb2 complex promotes B-cell activation can be considered.As the C-terminal SH3 domain of Grb2 constitutively interacts with SLP-65 (106), Grb2 may bring additional Ca^{2+} initiation complexes to the plasma membrane and directly to the activated BCR. Moreover, ITT-bound Grb2 is not available for signal inhibition via Dok-3 (see above) (92, 96). It is also likely that the presence of Grb2 stabilizes the 'open' conformation of the activated Igα/Igβ heterodimer (107). Despite the fact that the ITT in mIgG matches the consensus pYXXM docking site for the p85 regulatory subunit of PI3K, no evidence was obtained that PI3K activity contributes to ITT signaling (101). A likely explanation for the absence of PI3K signaling is that the positively charged arginine/lysine residue in the Y + 1 position of the ITT is not tolerated by SH2 domain of p85 but only by that of Grb2 (see above) (6). Collectively, the phosphorylated ITT and its association with Grb2 constitute a highly selective signaling module that provides BCR-intrinsic costimulation to mIgG and mIgE class-switched memory B cells and may render them less dependent on costimulation by helper T cells.

A positive regulatory role of Grb2 in BCR signaling is also suggested by the high affinity binding of the Grb2 SH2 domain to phospho-YXN sites in CD19 (108, 109), which in conjunction with the complement receptor 2 (CD21) and CD81 (TAPA) forms a prominent coreceptor complex for BCR

signal amplification (110). Consistent with the abnormal B-cell development and function in genetically engineered CD19 mouse mutants (111, 112), autoimmune diseases and primary antibody deficiency syndromes in human patients are caused by CD19 hyper- or hypofunction, respectively (113, 114).

The potent B-cell activation function of CD19 is accomplished by several SH2 domain-containing effector molecules that bind to CD19 in stimulation-dependent and in a constitutive manner (110). Formation of the signalosome requires Lyn, which phosphorylates CD19. Amplification of Ca^{2+} mobilization is achieved by direct PLC-γ2 recruitment and possibly also by binding of Vav isoforms, which provide GEF activity for small G proteins of the Rho/Rac family and have been implicated in Ca^{2+} flux regulation by the BCR. Vav isoforms can partially substitute for each other, but in the absence of all three Vav isoforms, BCR-evoked Ca^{2+} flux is completely abrogated (115). It has been demonstrated more recently that CD19, PLC-γ2, and Vav cooperate during formation of the immunological B-cell synapse and propagation of B-cell spreading (116, 117), most likely by reorganizing the actin cytoskeleton through activation of Rho/Rac-dependent pathways (118). CD19-associated Vav can also contribute to activation of PI3K and the Akt/PKB survival pathway (119). One key event for PI3K activation in B cells is CD19-mediated membrane translocation of the regulatory p85 subunit. A second event, which plays a synergistic and non-redundant role in PI3K activation, is association of p85 to the tyrosine-phosphorylated B-cell adapter for PI3K (BCAP) (120, 121). No PI3K activation is observed in mouse B cells that lack both CD19 and BCAP (122). How the BCAP/p85 complex is recruited to the plasma membrane and how exactly it feeds into pathway activation is unclear. Possibly phosphorylation of the YXN consensus motifs in BCAP initiates association with Grb2 and helps to target BCAP to its proper membrane destination. Grb2 may more directly contribute to CD19-mediated B-cell costimulation than it promotes B-cell inhibition via phosphatases (see above), i.e. by increasing the avidity of complex formation between CD19 and its effector through a second linkage for this effector at the coreceptor. A single Vav molecule may be anchored directly to CD19 through its own SH2 domain and indirectly through its SH3 domain, which forms an unusual dimer with the C-terminal SH3 domains of Grb2 (123–125) bound to CD19 via a pYXN motif. This multi-layered tethering mechanism boosts Ca^{2+} mobilization and/or formation of membrane microclusters. Consistent with this view, Vav localization in lipid rafts has been demonstrated to be controlled by Grb2 and SLP-65 (126), which itself is bound by the SH2 and SH3 domains of Grb2 (48, 49, 106). It can of course not be excluded that CD19-bound Grb2 recruits additional yet to be identified effector proteins to CD19. Clearly the discussed possibilities of how Grb2 supports CD19-mediated B-cell activation are not mutually exclusive.

A proteomic approach to elucidate the Grb2 interactome in activated B cells

In addition to the Grb2 interaction partners described above, other B-cell ligands have been detected by yeast-two-hybrid screenings or pull-down experiments with bacterially expressed fusion proteins. A plethora of Grb2 ligands has been described and functionally characterized in non-B-cell types including T cells. To obtain a comprehensive list of B-cell proteins that bind Grb2 *in vivo*, we employed stable isotope labeling by amino acids in cell culture (SILAC) in combination with high-end mass spectrometry analysis of purified protein complexes (127). This combination has been shown to be a powerful technique, not only for the quantification of proteins in complex samples (127) but also for the specific identification of protein interactions (128). The sensitivity and accuracy of this approach compares to that of co-immunoprecipitation experiments followed by Western blot analysis.

Murine Bal17.TR B cells, which lack endogenous Grb2 expression (63), were reconstituted with either wildtype Grb2 or with a Grb2 variant that harbors an C-terminal *One-Strep-Tag* (IBA BioTagnologies). The former transfectants were metabolically labeled in SILAC medium containing 'light' forms of the two amino acids lysine and arginine ($[^{12}C_6,^{14}N_2]$-Lys, $[^{12}C_6,\ ^{14}N_4]$-Arg). The latter transfectants, expressing tagged Grb2, were cultured with 'heavy' forms of lysine and arginine ($[^{2}D_4,^{12}C_6,^{14}N_2]$-Lys, $[^{13}C_6,^{14}N_4]$-Arg). To control for side effects of the labeling procedure, we also performed experiments in which cells were cultured in the converse manner. The differentially labeled cells were stimulated through their IgM-BCR for 5 min. Proteins were purified from the lysates with a *Strep-Tactin* column (IBA BioTagnologies) (129). The obtained protein fractions were pooled at a 1:1 ratio, separated by one-dimensional gel electrophoresis, and in-gel digested with endoproteinase trypsin. Peptide fragments were extracted and analyzed by liquid-chromatography-coupled tandem mass spectrometry (MS/MS) on an Orbitrap XL-mass spectrometer. The data were processed by the MaxQuant software (130, 131) to identify the corresponding proteins and to obtain accurate quantitative ratios for both samples. The quantification is made possible because the tryptic peptides derived from the differentially labeled cells can be distinguished in the mass spectrometer, owing to their specific mass shift that results from the distinct incorpo-

ration of heavy or light amino acids. The signal intensities of the peaks in the heavy versus light MS spectra (H/L ratio) correspond to the relative abundance of the peptides in each sample. This approach allows for discrimination between those proteins that non-specifically adhere to the matrix and those that are specifically affinity-purified and hence represent *in vivo* ligands for Grb2. The former can be detected by their appearance in both preparations a ratio of approximately 1:1. The peptide peak intensities of the affinity-purified Grb2 ligands are much higher in the sample preparation from cells expressing tagged Grb2 than in the negative control sample obtained from cells expressing untagged Grb2. As an example, *Fig. 1* shows an MS spectrum corresponding to the peptide EAISLVC$_{carb}$EAVPGAK derived from the Grb2 ligand SHC. This particular peptide was almost exclusively found in the sample preparation from Bal17.TR cells expressing tagged Grb2, which were in this case labeled with heavy amino acids. The corresponding light version of this SHC peptide was almost undetectable. *Table 1* shows the number of peptides identified for each ligand, the posterior error probability (PEP) of the identified proteins, and their relative abundance ratio for the two Bal17.TR transfectant lines cultured in SILAC medium in the combination light plus heavy and *vice versa*. The PEP together with the abundance ratio allows to unambiguously assign the identified protein as a Grb2 ligand in an unbiased manner. Our analysis is summarized in *Table 1*, and a comprehensive overview of the Grb2 interactome in B cells is depicted in *Fig. 2*.

The quantitative proteomic approach showed that the negative regulators SHIP1 and Dok-3 are the most frequently detected interacting proteins of Grb2 in Bal17.TR B cells. Protein tyrosine phosphatases are also prominently represented. In addition to SHP-1 and SHP-2, we detected the transmembrane phosphatase PTPRα and the cytosolic phosphatase PTP-PEST as novel B-cell ligands for Grb2. PTPRα comprises a short extracellular domain of 123 amino acids and two cytoplasmic phosphatase domains. Expression of PTPRα has not yet been described in B cells, but its interaction with the SH2 and C-terminal SH3 domain of Grb2 has been shown to occur in fibroblasts (132, 133). A wealth of evidence exists for the importance of PEST family phosphatases in immune cell activation and autoimmune inflammatory disorders (134).

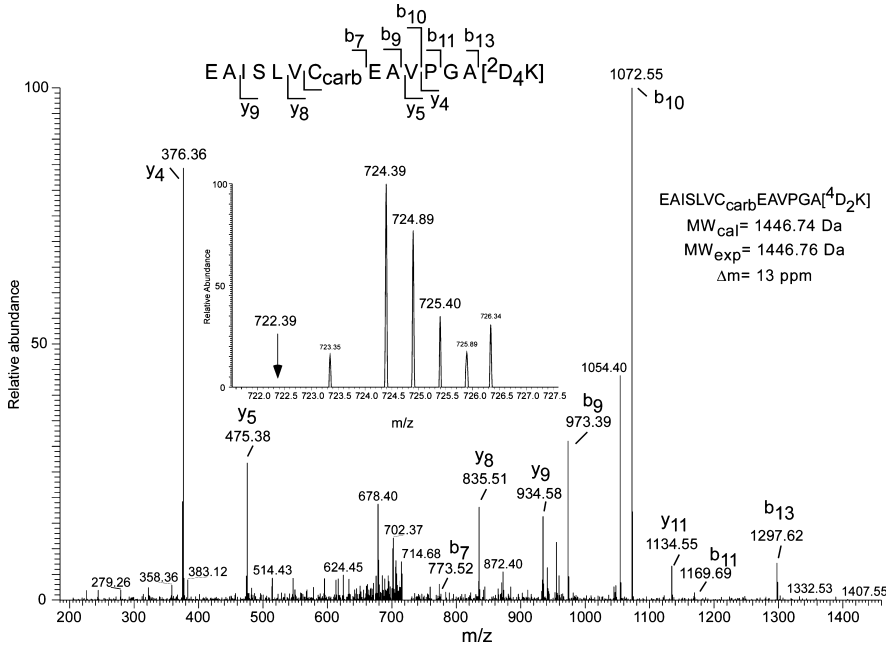

***Fig. 1.* Example of a mass spectrometric (MS) analysis for the identification of Grb2 ligands.** The depicted MS and MS/MS spectra are derived from tryptic peptides obtained from the mixture of proteins purified with tagged Grb2 and untagged Grb2 (negative control). The presence of the 'heavy' lysine (resulting in a mass shift of +4 atomic mass units) shows that the peptide is derived from cells which were SILAC-labeled with heavy amino acids and expressed (in this particular experiment) tagged Grb2. The peptide has a mass of 1446.76, is doubly charged, and has its mono-isotopic peak at a mass-to-charge ratio (m/z) of 724.39 in the full scan (central diagram). The m/z value of the corresponding peptide, which accommodates a 'light' lysine (+0) and is therefore derived from the sample preparation of cells expressing the untagged Grb2 version, is around 724.39. As this light peptide is below the detection limit, the protein that gave rise to the heavy peptide is unambiguously identified as being specifically affinity-purified with tagged Grb2and thus a Grb2 ligand. To deduce the amino acid sequence, the peptide was fragmented by collision-induced decay (CID), and fragments were recorded to give the MS/MS scan (below the full scan). The resulting fragment ions were searched through databases to eventually identify the peptide and assign it to SHC, in this case.

Table 1. **The reference list for Grb2 interactions in B cells**

PEP	Ratio H/L	No of peptides quant.	Acc. No.	Ligand	Function	Binding site (reported or predicted)	Grb2 domain	Reference
0	1/14.5	105	Q9ES52	SHIP1 (SH2 domain-containing inositol 5′-phosphatase 1)	inositol 5′-phosphatase	C-terminal PXXP sites	SH3 [C]	(82, 148)
0	42.8	81						
0	1/34.3	23	Q9QZK7	Dok-3 (downstream of kinase 3)	inhibitory adapter	pYXN	SH2	(96, 149)
10^{-208}	57.6	23						
0	1/24.0	74	Q62245	SOS1 (Son of Sevenless 1)	Ras GEF	3× PXXPXR 1× PPVPPRRRP	SH3 [N+C]	(28, 39, 45, 150, 151)
0	53.2	46						
0	1/19.7	3	P39054	dynamin 2	membrane scisson GTPase	unknown	SH3 [N]	this study and (152)
10^{-176}	17.0	3						
10^{-292}	1/20.6	32	P70218	HPK-1 (hematopoietic progenitor kinase 1)	MAP4K	unknown	SH3 [N+C]	(153–155)
10^{-156}	49.2	26						
10^{-277}	1/7.0	16	P22682	c-Cbl (cellular-Casitas B-lineage lymphoma)	E3 ubiquitin ligase	pYXN, PXXXRXXKP >1 pro-rich	SH3 + SH2	(141, 143, 144, 156)
10^{-99}	18.7	10						
10^{-271}	1/17.0	35	Q9QWY8	ASAP1 (ArfGAP-containing SH3 domain ankyrin repeats and PH domain 1)	Arf GTPase	2× pro-rich	SH3 [C]	this study and (157)
10^{-176}	30.0	26						
10^{-265}	1/13.3	48	Q02384	SOS2 (Son of Sevenless 2)	Ras GEF	4× pro-rich	SH3 [N+C]	this study and (158, 159)
10^{-50}	48.3	10						
10^{-252}	1/16.5	5	P98083	SHC (SH2-containing adapter)	cytosolic adapter protein	2× pYXN	SH2	(34, 38, 45, 156)
10^{-132}	68	5						
10^{-243}	1/22.0	2	Q3TTA7	Cbl-B (Casitas B-lineage lymphoma B)	E3 ubiquitin ligase	unknown	unknown	(160, 161)
10^{-120}	14.1	15						
10^{-239}	1/16.7	38	Q6P549	SHIP2 (SH2 domain-containing inositol 5′-phosphatase 2)	inositol 5′-phosphatase	unknown	SH3 [C]	(82)
10^{-184}	20.4	24						
10^{-236}	1/21.1	26	P18052	PTPRα (protein tyrosine phosphatase receptor typeα)	transmembrane protein tyrosine phosphatase	pYXN + ?	SH2 + SH3 [C]	(132, 162)
10^{-256}		27						this study
10^{-223}	1/32.9	26	P35831	PTP-PEST (protein tyrosine phosphatase- Pro-Glu-Ser-Thr)	protein tyrosine phosphatase	4× pro-rich	SH3 [N+C]	this study and (163)
10^{-124}	11.8	13						
10^{-190}	1/12.5	26	Q8BTI9	*PI3K-C2β (phosphoinositide 3-kinase C2β)	class II PI3K	2× pro-rich	SH3 [N+C]	this study and (164)
10^{-174}	1/14.3	18	O08908	p85-β	regulatory subunit of PI3K	unknown	unknown	this study
10^{-193}	8.5	19						
10^{-139}	1/19.8	11	P35235	SHP-2 (SH2 domain-containing protein phosphatase 2)	protein tyrosine phosphatase	pYXN, pro-rich	SH2 and SH3 [C]	(90, 91)
10^{-51}	12.2	10						
10^{-99}	1/7.3	9	Q91ZR2	SNX18 (sorting nexin 18)	cytosolic adapter protein	unknown	unknown	this study
10^{-71}	9.2	13						
10^{-87}	1/7.5	19	P26450	p85-α	regulatory subunit of PI3K	2× pro-rich	SH3 [N+C]	this study and (165)
	8.4	17						
10^{-79}	1/50.4	2	Q8C0D4	ARHGAP12 (RhoGTPase-activating protein 12)	Rho GAP	unknown	unknown	this study
10^{-91}	9.4	1						
10^{-74}	1/7.3	5	P25918	CD19	forms complex with CD21 (CR2) and CD81, activating BCR coreceptor	6× pYXN	SH2	(108, 109)
10^{-55}	39.6	7						
10^{-73}	1/21.1	10	Q9EQ32	BCAP (B cell adapter for PI3K)	cytosolic adapter protein for PI3K activation	pYXN (?)	SH2 (?)	this study
10^{-59}	17.9	10						
10^{-43}	17.1	8	Q9Z1S8	*Gab2 (Grb2-associated binder)	cytosolic adapter	PXXXRXXKP	SH3 [C]	(10, 166, 167)
10^{-38}	1/15.9	7	P27870	Vav1 (hebrew: for 6th oncogene)	Rho/Rac GEF	SH3	SH3 [C]	(123, 125, 168)

Table 1. (Continued)

PEP	Ratio H/L	No of peptides quant.	Acc. No.	Ligand	Function	Binding site (reported or predicted)	Grb2 domain	Reference
10^{-61}	32.7	12						
10^{-35}	1/66.7	8	P29351	*SHP-1 (SH2 domain-containing protein phosphatase 2)	protein tyrosine kinase	unknown	SH3	(169)
10^{-32}	1/7.0	19	Q9QUN3	SLP-65 (SH2 domain-containing leukocyte protein of 65 kDa)	cytosolic adapter	1× pYXN + 2× PXXXRXXKP	SH2 + SH3 [C]	(48, 49, 106)
10^{-52}	31.6	15						
10^{-23}	1/21.9	4	P21855	CD72	C-type lectin,inhibitory BCR co-receptor	1× pYXN	SH2	(88, 89)
10^{-27}	33.4	6						
10^{-22}	1/15.4	1	Q8BZ98	dynamin 3	membrane scisson GTPases	Unknown	unknown	this study
10^{-16}	52.8	1						
–				mIgG/E (membrane–bound IgG/E)	BCR-intrinsic signal boost for class-switched B cells	pYRNM (IgG)pYXNX (IgE)	SH2	(101)
–				FcγRIIb (Fc γ receptor IIb)	low affinity receptor for IgG inhibitory BCR coreceptor	1× pYXN	SH2	(170)
–			.	CD22	Sialic acid-binding Ig lectin inhibitory BCR coreceptor	1× pYXN	SH2	(86, 87)
–				NTAL (non-T-cell activation linker)	transmembrane adapter	5× pYXN	SH2	(92, 171)
–				Lax (linker of activated X cells)	transmembrane adapter	5× pYXN	SH2	(172)
–				GAPT (Grb2-binding adapter protein, transmembrane)	transmembrane adapter	1× pro-rich	SH3	(173)
–				HS1 (hematopoietic-specific substrate 1)	cytosolic adapter protein	2× pro-rich	SH3 [N]	(174)

Proteins co-purified with *One-Strep-Tag-modified* Grb2 from SILAC-labeled B cells and identified by mass spectrometric analysis are listed according to their protein-PEP (posterior error probability). The protein-PEP is a parameter for the probability of random identification in database searches and calculated by multiplication of the PEPs of all identified peptides derived from a single protein (for details see (130). MS analyses were performed from two experiments reflected by two PEP values and two H/L ratios. The ratio H (Heavy) versus L (Light) indicates the relative abundance of the proteins in the MS analysis. The first MS analysis (1° H/L ratio) was performed with tagged Grb2 labeled by 'light' amino acids, and with untagged Grb2 labeld by 'heavy' amino acids. In the second MS analyis, (2° H/L ratio) the labeling of tagged and untagged Grb2 was invers i.e., the former carried 'heavy' amino acids and the latter 'light' amino acids. The number of quantified peptides are indicated for each protein. Grb2 ligands identified in only one purification are marked with an asterisk. Proteins that have been shown to interact with Grb2 in B cells before but were not identified in our MS approach are listed at the end without MS-specific values. Binding sites are shown in single letter code for amino acid where × denotes any amino acid.

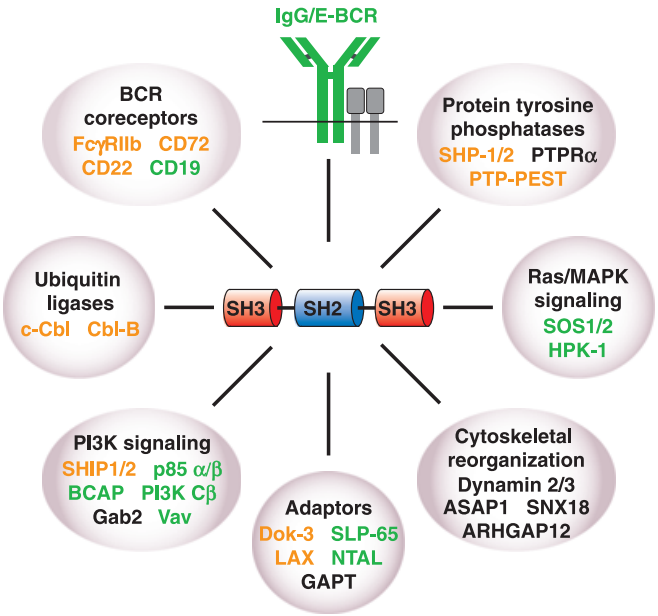

Fig. 2. The interactome of Grb2 in B cells. The SH2 and SH3 domains of Grb2 (center) interact with positive (green) and negative (orange) signal regulators of different pathways.

The proto-oncoprotein and E3 ubiquitin ligase Cbl (for Casitas B-lineage lymphoma) together with Cbl-B are two other well-known inhibitors of B-cell activation (135, 136), and both were detected frequently as Grb2 binders in our SILAC/MS approach. Cbl proteins control the induction of B-cell tolerance by adjusting the BCR signaling threshold through ubiquitinylation of Syk and its concomitant targeting to proteolytic degradation (137–139). Furthermore, Cbl activity regulates proteolysis of the antigen-ligated BCR complex following its internalization (140). Cbl and Grb2 have been co-immunoprecipitated from B-cell lysates (141), but the molecular details of this interaction have been worked out in other cell types and for Gads and GrpL (142–144).

Under the stimulation conditions utilized, the negative regulatory coreceptors FcγRIIb and CD22 were not detected in our proteomic screen. Lack of the former is explained by the absence of co-crosslinking FcγRIIb with the BCR, which is required for efficient Grb2 recruitment (see above). Expression of CD22 in Bal17.TR B cells needs to be confirmed. Phosphorylated mIgG and mIgE were missed, because Bal17.TR cells express an IgM-BCR. The Grb2 binder NTAL and LAX are lipid raft residents, which, dependent on the detergent conditions, are sometimes difficult to solubilize and hence may be lost during the purification procedure. The Grb2 ligands that were absent in our analysis are listed at the end of *Table 1* without a PEP value.

Among the novel and frequently detected B-cell ligands of Grb2 are cytoskeleton components implicated in actin reorganization and vesicle trafficking (*Table 1*). The association of Grb2 with SOS1 has been extensively discussed above, and the prominent presence of SOS1, SOS2, and the hematopoietic progenitor kinase-1 (HPK-1) in our analysis may reflect the involvement of these complexes in activation of the Ras/MAPK pathway by other receptors than the BCR. Elucidation of the Grb2 interactome in Bal17.TR B cells confirmed a prominent role of Grb2 in PI3K regulation. Grb2 associated with the enzyme's components themselves, the regulatory subunits p85α and β and the catalytic isoform Cβ, as well as with the two key activators CD19 and BCAP, which cooperate for full PI3K activation in B cells. Almost certainly Grb2 is directly or indirectly involved in the primary step of PI3K activation, i.e., in membrane translocation of the p85 subunits. However and as described above, the most frequently detected Grb2 ligand is SHIP, which is a main antagonist of PI3K. This apparent discrepancy is not easily explained at a mechanistic level. It however suggests the existence of distinct cellular Grb2 pools that navigate along discrete pathways without crosstalk. This conclusion again emphasizes the importance of precise subcellular localization for signaling homeostasis.

Concluding remarks

Despite the remarkably simple architecture, Grb2 is one of the most versatile proteins in signal transduction. Currently, 194 interaction partners of Grb2 are listed in the Human Protein

Reference Database (145), and 515 Grb2 ligands are described in the IntAct database (146). It is thus easy to comprehend the embryonic lethality upon global loss of Grb2 expression in mice (147). B-cell-specific ablation of Grb2 expression in the mouse has not yet been described, but the multiple roles of Grb2 in positive and negative regulatory cascades suggest indispensable contributions of Grb2 for proper signal integration to induce and maintain B-cell tolerance. Some Grb2 functions however may be redundant and replaced in the case of Grb2 deficiency by other Grb2 family members such as Gads. Deciphering the Grb2 network at a molecular level is a difficult task but will be facilitated by novel proteomic techniques to unravel protein-protein interactions *in vivo*.

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