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Expression of the Grb2-Related Protein of the Lymphoid System in B Cell Subsets Enhances B Cell Antigen Receptor Signaling Through Mitogen-Activated Protein Kinase Pathways

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Adaptor proteins play a critical role in regulating signals triggered by Ag receptor cross-linking. These small molecules link receptor proximal events with downstream signaling pathways. In this study, we explore the expression and function of the Grb2-related protein of the lymphoid system (GrpL)/Grb2-related adaptor downstream of Shc adapter protein in human B cells. GrpL is expressed in naïve B cells and is down-regulated following B cell Ag receptor ligation. By contrast, germinal center and memory B cells express little or no GrpL. Using human B cell lines, we detected constitutive interactions between GrpL and B cell linker protein, Src homology (SH)2 domain-containing leukocyte protein of 76 kDa, hemopoietic progenitor kinase 1, and c-Cbl. The N-terminal SH3 domain of GrpL binds c-Cbl while the C-terminal SH3 domain binds B cell linker protein and SH2 domain-containing leukocyte protein of 76 kDa. Exogenous expression of GrpL in a GrpL-negative B cell line leads to enhanced Ag receptor-induced extracellular signal-related kinase and p38 mitogen-activated protein kinase phosphorylation. Thus, GrpL expression in human B cell subsets appears to regulate Ag receptor-mediated signaling events. The Journal of Immunology, 2003, 170: 349–355.

B cell Ag receptor (BCR)3 ligation can lead to a number of different cell fates. The developmental stage of the B cell is one factor that dictates cellular outcomes (reviewed in Refs. 1–3). At the immature and transitional 1 (T1) stages of B cell development, B cells expressing a BCR complex specific for autoantigens are negatively selected. At these stages, BCR ligation can induce apoptosis. By contrast, at the transitional 2 (T2) stage of B cell development, BCR ligation induces B cell proliferation (4). BCR expression is also critical for mature B cell survival (5), indicating the importance of a basal level of signaling. However, BCR ligation can exclude B cells from follicles in a manner dependent on the concentration of the Ag and the availability of T cell help (6–8).

The nature of the downstream signaling events induced by BCR ligation dictates the outcome of stimulation. BCR ligation results in the activation of protein tyrosine kinases, such as Syk, Lyn, and Btk (1). How these kinases are coupled to downstream effector molecules may determine cell fate. A group of proteins critical for linking signaling enzymes, such as kinases, with their substrates are called adaptor proteins, molecules that possess no intrinsic enzymatic activity and consist of molecular binding domains. Adaptor proteins can be as important to enzymatic processes as the enzymes themselves. For example, patients with mutations in the B cell adapter protein B cell linker protein (BLNK) show immune deficiencies nearly identical with those with mutant forms of Btk (9).

In this study, we researched a member of the Grb2 family of adapter proteins cloned in our laboratory and others, which we call Grb2-related protein of the lymphoid system (GrpL), also known as Gads, Mona, Grf40, and Grb2-related protein with insert domain (10–14). GrpL/Grb2-related adaptor downstream of Shc (Gads) consists of an N-terminal Src homology (SH)3 domain, an SH2 domain, a proline/glutamine-rich region, and a C-terminal SH3 domain. The function of GrpL has been most extensively studied in T cells where it provides the physical and functional link between linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) (10, 13, 15). Following ligation of the TCR, the SH2 domain of GrpL binds tyrosine-phosphorylated LAT. The C-terminal SH3 domain of GrpL constitutively binds SLP-76. The recruitment of GrpL to LAT, then, brings SLP-76 into the TCR signaling complex. These interactions are critical for the regulation of calcium signaling in T cells (16).

Relatively little is known about the expression and function of GrpL in B cells, although GrpL is expressed in certain B cell lines (10). In this study, we demonstrate that GrpL is expressed in human naïve B cells and expression is down-regulated when B cells are activated. GrpL constitutively interacts with BLNK, SLP-76, hemopoietic progenitor kinase 1 (HPK1), and c-Cbl in B cell lines. Furthermore, exogenous GrpL expression in a GrpL-negative human B cell line enhances BCR-induced activation of extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase (MAPK). Thus, GrpL may function in B cells to regulate signaling through the BCR.
Materials and Methods

Cell and Abs

The human B cell lines MP-1 and BJAB were grown in RPMI 1640 medium containing 10% FCS, nonessential amino acids, sodium pyruvate, penicillin, streptomycin, and glutamine. Human dense tonsillar B cells and T cells were prepared as described previously (17). Briefly, lymphocytes were incubated with SRBC. The mixture was separated using Ficoll. The upper B cell layer and the lower T cell/RBC layer were isolated. Red cells were lysed in 0.15 M NH4Cl, 1.0 mM KHCO3, and 0.1 mM Na2EDTA (pH 7.2). The UW40 IgG2a mAb specific for GrpL was prepared as described (18). Biotinylated anti-GrpL and IgG2a control mAb were prepared by dialyzing against NaHCO3 overnight, incubating with biotin-O-succinimidyl ester for 4 h, and then dialyzing against PBS overnight. PerCP-conjugated anti-CD3, APC-conjugated anti-CD19, FITC-conjugated anti-IgD, and streptavidin-PE were purchased from BD PharMingen. Cells were analyzed using a FACSCalibur (BD Immunocytometry Systems). RNA was isolated from cells using TRIzol according to the product recommended protocol followed by PCR with One-Tube RT-PCR system (Roche, Indianapolis, IN) or indirectly by DNA polymerase (Life Technologies). The following primers were used: GrpL forward primers, GCG TCT AGA GCC ACC ACC ATG GAA GCT GTT GCC AAG TTT or CTC ACC CCC TTC TTC TCA AAA AGG TTC ACG; GrpL reverse primers, CGT CAT CCT CCA GGG CCT CAA AGT C or CTG GGA CCG CCG CTC GAG CACT GGT GTT GCC G3PDH forward primer, TGA AGG TCG GCG TCA ACG GAT TTG GT; and G3PDH reverse primer, CAT GTG GGC CAT GAG GTC CAC CAC.

In vitro kinase assay and reimmunoprecipitation

In vitro kinase assays and reimmunoprecipitation were performed as described previously (19). Briefly, anti-GrpL immune complexes were incubated with [γ-32P]ATP and separated by SDS-PAGE. After autoradiography of the dried gel, bands were excised from the gel and the protein was eluted and incubated with Abs coupled to protein A-Sepharose. The isolated protein was then separated by SDS-PAGE and visualized by autoradiography.

cDNA and mutagenesis
cDNA expressing myc-tagged, wild-type GrpL (wt-GrpL) in the pcDNA3.1+ Myc-His A vector (Invitrogen, Carlsbad, CA) was prepared as described previously (10). Mutagenesis of wt-GrpL to generate N-SH3–GrpL and C-SH3–GrpL was performed using the QuickChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). To generate stable cell lines, 107 BJAB B cells were electroporated with 20 μg pcDNA GrpL (240 V, 960 μF, using Gene-Pulsar (Bio-Rad, Hercules, CA)). After 24 h, the cells were divided into 12-well plates and incubated with 3 mg/ml G418 (Calbiochem, San Diego, CA). Clones were screened by Western blot and repeatedly subcloned and screened to achieve clonal populations.

Calcin assays

Cells were loaded with indo-1 at 37°C for 45 min. Calcium was measured by flow cytometry on a BD LSR system (BD Immunocytometry Systems).

Results

Expression of GrpL in human B cell subsets

Previously, we reported that GrpL/Gads is expressed in human B cell lines (10). In this study, we further examined GrpL expression in B cells by staining human tonsillar lymphocytes with cell surface markers followed by intracellular staining with anti-GrpL or control mAb (Fig. 1A). We detected two distinct GrpL-positive populations of lymphocytes (Fig. 1A). Each population was then analyzed for CD19 and CD3 expression. GrpLhigh cells were nearly exclusively CD3+ while GrpLlow cells were predominantly CD19+. Similar results were obtained when enriched T and B cells were lysed and analyzed by Western blot (Fig. 1A). Thus, both B and T cells express GrpL, and T cells express GrpL at higher levels than do B cells.

B cells can be subdivided into naive, germinal center (GC), and memory B cells based on IgD and CD38 expression (20). Naive B cells (CD19+IgD+CD38−) express GrpL, but GC B cells (CD19+IgD−CD38−) express little or no GrpL (Fig. 1B). Memory B cells, enriched in the CD19+IgD−CD38− population, are also GrpL-negative. The isotype control in GC and memory cells appears brighter than in naive B cells because large activated cells nonspecifically adsorb more protein than do small resting cells. RT-PCR analysis of sorted naive and GC B cells showed that GrpL mRNA is expressed in naive B cells, but not in GC B cells (Fig. 1B, inset). Thus, the regulation of GrpL expression during B cell activation appears to take place at the level of either transcription or mRNA stability.

Naive B cells can be activated through CD40 or through the BCR. Thus, we tested whether either of these signals can regulate GrpL expression in dense tonsillar B cells. B cells were incubated overnight with medium alone, with anti-CD40, or with anti-IgM. Anti-IgM treatment down-regulated GrpL mRNA and protein expression, but anti-CD40 did not (Fig. 1C). By contrast, in the same cells, CD40 ligation up-regulated expression of the Bam32 adapter protein and anti-IgM did not (21).

In summary, there are GrpL+ and GrpL− B cell subsets. GrpL is expressed in mature naive B cells. Following BCR ligation and entry into GCs, GrpL expression is down-regulated in GC B cells and remains “off” in memory B cells.

GrpL associates with BCR signaling proteins

We next defined which proteins associate with GrpL/Gads in B cells using the human B cell line MP-1. Shortly after BCR ligation, a number of protein kinases become active and new tyrosine and serine/threonine phosphorylation can be detected on many substrates. If GrpL plays a role in BCR signaling, it would probably associate with tyrosine-phosphorylated proteins or a kinase activity. To test these possibilities, lysates from resting or anti-IgM-stimulated MP-1 B cells were immunoprecipitated with anti-GrpL or control mAb. In one set of experiments, tyrosine phosphorylation of GrpL-associated proteins was analyzed by anti-phosphotyrosine Western blotting (Fig. 2A). In other experiments, anti-GrpL immune complexes were incubated with [γ-32P]ATP and resulting phosphoproteins were visualized by autoradiography (Fig. 2B). GrpL-associated molecules that were inductibly tyrosine phosphorylated included ERK and p38 MAPK.
phosphorylated include bands at 120, 76, and 55 kDa (Fig. 2A). The immune complex kinase assay revealed a constitutive association between GrpL and a kinase activity. The most prominent in vitro substrates for the GrpL-associated kinase activity were 120, 95, 76, and 38 kDa in size (Fig. 2B). Thus, GrpL does bind proteins that are inducibly tyrosine phosphorylated upon BCR ligation and interacts with an inducible kinase activity.

The four prominent phosphoproteins in the immune complex kinase assay were then excised from the gel, purified, and reimmunoprecipitated with Abs against candidate proteins. The four major proteins in B cell-derived anti-GrpL complexes were GrpL itself, the SLP-76 adapter, the HPK1 serine/threonine kinase, and the c-Cbl ubiquitin ligase (Fig. 2C). Two of the heavily tyrosine-phosphorylated proteins seen in Fig. 2A, 120 and 76 kDa, comigrated with CD3 expression. Western blot, Lysates from either unsorted lymphocytes (U) or enriched T or enriched B cells were Western blotted with anti-GrpL. The GrpL-negative B cell line BJAB is a negative control. B, Human tonsillar lymphocytes were stained with anti-CD19, anti-CD3, and either anti-GrpL (solid line) or isotype control (dotted line). GrpLbright and GrpLdull cells were analyzed for CD19 and CD3 expression. Western blot, Lysates from either unsorted lymphocytes (U) or enriched T or enriched B cells were Western blotted with anti-GrpL. The GrpL-negative B cell line BJAB is a negative control. B, Human tonsillar lymphocytes were stained with anti-CD19, anti-CD3, anti-CD38, and either anti-GrpL or isotype control. Naive, GC, and memory B cells were analyzed for GrpL expression by intracellular staining. Inset, RT-PCR analysis on unsorted B cells (lane 1), sorted naive B cells (lane 2), and sorted GC B cells (lane 3). C, Enriched naive tonsillar B cells were incubated overnight with medium alone, anti-CD40, or anti-IgM. GrpL expression was measured by intracellular staining with anti-GrpL or by RT-PCR. The expression of GrpL was determined by semiquantitative PCR of 1/1, 1/10, and 1/100 dilutions of GrpL and by PCR of 1/100, 1/1,000, and 1/10,000 dilutions of G3PDH. The relative expression of GrpL was measured by phospho-image quantitation of the 1/10 dilution of GrpL relative to that of the 1/100 dilution of G3PDH.
c-Cbl and SLP-76. However, the bands comigrating with GrpL and HPK1 were barely detectable in the anti-phosphotyrosine Western blot. The 55-kDa protein seen in the anti-phosphotyrosine Western blot was faint in the kinase assay.

To further assess the interactions between GrpL and SLP-76, HPK1, and c-Cbl, lysates from MP-1 B cells were immunoprecipitated with anti-GrpL, antisera against SLP-76, HPK1, or c-Cbl, or the appropriate isotype control. Resulting immune complexes were Western blotted with anti-GrpL. (Fig. 3). GrpL constitutively associates with SLP-76, HPK1, and c-Cbl. The faint GrpL band in these experiments is reflective of the fact that MP-1 B cells, like primary B cells, have relatively low levels of GrpL expression, as seen by intracellular staining and FACS analysis (data not shown). Immunoprecipitation studies also revealed a constitutive interaction between GrpL and the SLP-76 homolog BLNK in both GrpL-expressing BJAB B cells (Fig. 4) and MP-1 B cells (data not shown).

The effect of mutating the SH3 domains of GrpL on protein interactions

To explore the nature of the interactions between GrpL and BLNK, SLP-76, HPK1, and c-Cbl, we generated stable cell lines using the GrpL-negative BJAB human B cell line. Cell lines created express either wt-GrpL or GrpL with point mutations that disrupt the function of the SH3 domains. Mutation of tryptophan 34 to lysine (N-SH3* -GrpL) inactivates the N-terminal SH3 domain and mutation of tryptophan 308 to lysine (C-SH3* -GrpL) inactivates the C-terminal SH3. These tryptophan residues align with amino acids in Grb2 that are essential for the function of the SH3 domains of Grb2 (22). These cell lines express similar levels of GrpL protein as measured by Western blot (Fig. 4, bottom) or by intracellular staining (data not shown). The interaction between GrpL and c-Cbl was dependent on the N-terminal SH3 domain of GrpL. In contrast, the interactions between GrpL and BLNK and GrpL and SLP-76 depended on the C-terminal SH3 domain of GrpL. In the case of HPK1, dependence was on the N-terminal SH3 domain of GrpL with either the N-terminal or C-terminal SH3 domain was mutated, indicating that HPK1 may bind either SH3 domain or by another mechanism.

Exogenous expression of GrpL in GrpL-negative B cells augments BCR signaling

Using the stable cell lines described above, we investigated the effects of exogenous GrpL expression on downstream signaling events. Unlike in T cells where loss of GrpL expression abolished calcium signaling (16), wt-GrpL expression in B cells did not appear to alter calcium influx significantly (Fig. 5). GrpL-negative and GrpL-positive BJAB B cells yielded similar calcium responses over a range of doses of anti-IgM. Mutation of either SH3 domain did slightly impair BCR-mediated calcium influx. This inhibition was evident only at low doses of anti-IgM. However, GrpL expression did augment signaling through the MAPK pathways. Expression of wt-GrpL in BJAB B cells increased basal and BCR-induced ERK phosphorylation at all doses of anti-IgM used (Fig. 6, A and C). This enhanced ERK activation did not require a functional N-terminal SH3 domain as expression of N-SH3*-GrpL also resulted in markedly enhanced ERK phosphorylation (Fig. 6, B and C). Mutation of the C-terminal SH3 domain partially inhibited the GrpL-induced increase in ERK phosphorylation. Like ERK, p38 MAPK phosphorylation was also enhanced by the expression of wt-GrpL (Fig. 6, A and D). This increase was
independent of the C-terminal SH3 domain. The role of the N-terminal SH3 domain is less clear due to the variability of the assay. Expression of wt-GrpL only modestly augmented BCR-induced c-Jun N-terminal kinase activation, and it was unclear whether mutation of the SH3 domains affected the modest changes in c-Jun N-terminal kinase signaling (data not shown).

**Discussion**

In these studies, we report that there are GrpL-positive and GrpL-negative human B cell subsets. GrpL/Gads expression in B cells leads to enhanced phosphorylation of ERK and p38 MAPK (Fig. 6), pathways leading to enhanced cell survival and proliferation (23, 24). Thus, GrpL expression may initially contribute to the survival and clonal expansion of naive B cells in response to antigenic stimulation. Antigenic stimulation also leads to the downregulation of GrpL mRNA and protein (Fig. 1, B and C). Differences in GrpL expression between naive and GC B cells may contribute to the differences in cellular outcome seen in these subsets. Whereas naive human B cells proliferate in response to BCR ligation, GC B cells may undergo apoptosis after BCR stimulation (25). GC B cell survival may require T cell-dependent factors, such as CD154 (CD40 ligand) and IL-2 (26). Thus, GrpL expression in naive B cells may facilitate recruitment of the B cell into a T cell-dependent immune response. Subsequently, GrpL downregulation may ensure that the appropriate extracellular environment, such as T cell help, is present before differentiation occurs. Memory B cells, despite the lack of GrpL, proliferate in response to anti-IgM (25). By contrast, the Bam32 adapter protein, which regulates B cell fate, is highly expressed in GC B cells and is up-regulated by CD40 ligation and BCR ligation (21, 27). Thus, B cells use distinct adapter proteins at different stages of differentiation to regulate BCR signaling.

Exogenous GrpL expression in a GrpL-negative B cell line augments phosphorylation of ERK and p38 MAPK (Fig. 6). This is similar to the role of GrpL in T cells and macrophages. Transgenic expression of a dominant negative GrpL construct in murine T cells inhibits TCR-induced ERK activation (28). Likewise, overexpression of GrpL in a myeloid cell line enhances ERK activity in response to M-CSF (29). In B cells, GrpL expression is not required for the activation of ERK and p38 MAPK. Instead, GrpL may provide an additional mechanism for the recruitment and activation of signaling molecules upstream of these serine/threonine kinases. For example, there are at least two pathways that contribute to the activation of ERK. Genetic studies in the DT40 system show phospholipase C (PLC)-β2 and Ras are each partially required for ERK activity (30). Ras is likely activated after recruitment of Grb2 to proteins such as CD22 and CD72 (31, 32). In contrast, PLC-γ2 is activated following its recruitment to BLNK.

**FIGURE 5.** Mutation of either GrpL SH3 domain inhibits calcium influx at low doses of anti-IgM. Stable cell lines expressing wt-GrpL, N-SH3*-GrpL, or C-SH3*-GrpL or nontransfected BJAB B cells were loaded with indo-1 and stimulated with the indicated doses of anti-IgM.

**FIGURE 6.** Exogenous GrpL expression in a GrpL-negative B cell line enhances BCR-induced activation of MAPK pathways. A: Nontransfected BJAB or stable cell lines expressing wt-GrpL or C-SH3*-GrpL cells were stimulated with 0, 0.1, 1.0, or 10 μg/ml anti-IgM. Lysates were Western blotted with anti-phospho-p38 and anti-phospho-ERK. Membranes were stripped, blotted with anti-p38 MAPK, stripped again, and blotted with anti-GrpL. B: Nontransfected BJAB or cell lines expressing N-SH3*-GrpL were treated and Western blotted as in A. C and D: Relative band intensities in experiments represented by A and B were quantitated using NIH Image. The ratio of the phospho-specific band compared with the p38 MAPK loading control was calculated and normalized to unstimulated nontransfected BJAB cells. Each bar represents the mean ± SEM calculated based on the results of between 3 and 18 experiments. □, Unstimulated; ■, 1 ng/ml; ■, 10 ng/ml; ■, 100 ng/ml; □, 1 μg/ml; and □, 10 μg/ml.
In the absence of GrpL, the BLNK homolog may associate with the BCR signaling complex via an interaction with Igα or other proteins (33, 34). In the presence of GrpL, the interaction between BLNK and the C-terminal SH3 domain of GrpL (Fig. 4) provides an additional mechanism by which BLNK associates with the signaling complex and couples to ERK activation.

The BLNK homolog SLP-76 has not been well studied in B cells. In this study, we have demonstrated SLP-76 expression in the MP-1 and BIAB cell lines, and others have described SLP-76 expression in other B cell lines (35, 36). Both BLNK and SLP-76 are found in bone marrow-derived macrophages (37). Hence, both BLNK and SLP-76 may be coexpressed in primary B cell subsets.

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BLNK and the C-terminal SH3 domain of GrpL (Fig. 4) provides proteins (33, 34). In the presence of GrpL, the interaction between... phosphorylation. GrpL-negative B cells enhance phosphorylation of ERK and p38 MAPK.

References


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