GrpL, a Grb2-related Adaptor Protein, Interacts with SLP-76 to Regulate Nuclear Factor of Activated T Cell Activation

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Summary

Propagation of signals from the T cell antigen receptor (TCR) involves a number of adaptor molecules. SH2 domain–containing protein 76 (SLP-76) interacts with the guanine nucleotide exchange factor Vav to activate the nuclear factor of activated cells (NF-AT), and its expression is required for normal T cell development. We report the cloning and characterization of a novel Grb2-like adaptor molecule designated as Grb2-related protein of the lymphoid system (GrpL). Expression of GrpL is restricted to hematopoietic tissues, and it is distinguished from Grb2 by having a proline-rich region. GrpL can be coimmunoprecipitated with SLP-76 but not with Sos1 or Sos2 from Jurkat cell lysates. In contrast, Grb2 can be coimmunoprecipitated with Sos1 and Sos2 but not with SLP-76. Moreover, tyrosine-phosphorylated LAT/pp36/38 in detergent lysates prepared from anti-CD3 stimulated T cells associated with Grb2 but not GrpL. These data reveal the presence of distinct complexes involving GrpL and Grb2 in T cells. A functional role of the GrpL–SLP-76 complex is suggested by the ability of GrpL to act alone or in concert with SLP-76 to augment NF-AT activation in Jurkat T cells.

Key words: GrpL • SLP-76 • Grb2 • nuclear factor of activated cells • cell activation

Ligation of the T cell antigen receptor (TCR) complex activates protein tyrosine kinases (PTKs)† including the Src family PTKs Fyn and Lck to phosphorylate immunoreceptor tyrosine-based motifs (ITAMs) within associating CD3 chains. This leads to the recruitment and activation of the Syk family kinase ZAP-70 and possibly other proteins such as Shc to phosphorylated CD3ζ. These activated PTKs in turn phosphorylate a variety of proteins, and thereby couple TCR ligation to a number of intracellular signaling cascades culminating in T cell activation. Principal downstream substrates/effectors for TCR-activated PTKs have been identified. They include phospholipase C-γ1 (1-3), the 85-kD subunit of the phosphatidylinositol 3-kinase (4), the src homology (SH)2 domain–containing leukocyte protein of 76 kD (SLP-76) (5, 6), the proto-oncogene product Cbl (5, 7), the Vav guanine nucleotide exchange factor (GEF) of the Rho/Rac/CDC42 family of GTP-binding proteins (8, 9), and the recently cloned and characterized linker for activation of T cells (LAT) (10–14). Functional interactions between these phosphorylated effector molecules are believed to be necessary for the operation of major pathways required for T cell maturation including (a) intracellular free Ca2+ release, (b) activation of the nuclear factor of activated cells (NF-AT), and (c) mitogen-activated protein kinase (MAPK) activation. These events ultimately converge to induce gene expression, proliferation, or differentiation of T cells (15–17).

SLP-76 is an essential adaptor molecule in T cells. When it is tyrosine phosphorylated, probably by ZAP-70, SLP-76 may become a ligand for Vav (9, 18, 19). In Jurkat cells, the tyrosine-phosphorylated SLP-76–Vav complex augments TCR-induced activation of NF-AT, and hence expression of IL-2 (9, 18–21). The in vivo function of SLP-76 in T lineage cells is further illustrated by recent studies of SLP-76-deficient mice. Mice deficient in SLP-76 expression show profound defects in T cell development, and contain no CD4/CD8 double positive thymocytes, resulting in a complete loss of peripheral T cells (22, 23).

1Abbreviations used in this paper: GEF, guanine nucleotide exchange factor; GrpL, Grb2-related protein of the lymphoid system; IP, immunoprecipitate; MAPK, mitogen-activated protein kinase; NF-AT, nuclear factor of activated T cells; PTK, protein tyrosine kinase; SH, src homology; SHP, SH2 domain–containing protein tyrosine phosphatase; SLP-76, SH2 domain–containing leukocyte protein of 76 kD.
One connection between the TCR and SLP-76 may be provided by Grb2 (24). The SH2 domain of Grb2 binds to both Shc and LAT/pp36/38, allowing Grb2 to be localized to the plasma membrane where most of the TCR-activated PTKs are (13, 14, 25). The SH3 domain(s) of Grb2 bind to SoS1 and SoS2 (GEF of the Ras family of GTP-binding protein) (26), SLP-76 (21, 27), and Cbl (7, 28, 29). The recruitment of SoS to the plasma membrane by Grb2 initiates the MAPK pathway, whereas the recruitment of SLP-76 (30), Vav (8, 9), and Cbl (7, 31, 32) to the plasma membrane probably facilitates their phosphorylation by TCR-activated PTK.

The studies above suggest that Grb2 may function to link multiple downstream effector molecules, e.g., SLP-76, to LAT and possibly to the vicinity of the activated TCR complex. However, more recent studies have revealed the presence of an additional Grb2-like adaptor molecule, Grap, which has the SH3-SH2-SH3 organization as Grb2 (33, 34). Interestingly, expression of Grap appears to be relatively restricted to hematopoietic tissues (33, 34). Besides binding to c-kit, the Bcr-Abl oncoprotein, and the erythropoietin receptor, Grap also interacts with LAT/pp36/38, binding to c-kit, the Bcr-Abl oncoprotein, and the erythropoietin receptor, Grap also interacts with LAT/pp36/38, the SH2 domains of GrpL using the full-length GrpL cDNA as template and the following primer pairs forward primer, 5'-GGG ATG TGG ACC TCC CAG TTT CCC AAA TGG TTT CAC; reverse primer, 5'-GGG GGG AAT TCC TCT TGT GTC TCT TCT AAG GAA GAT CTC G. Underlined nucleotides represent BamHI and EcoRI sites in the forward and reverse primers, respectively. PCR products were cut with these enzymes and ligated into BamHI/EcoRI sites in the forward and reverse vectors, respectively. The PCR product was cloned into the pCDNA3.1(+) (Invitrogen), in-frame with the c-Myc epitope and poly-His tags. To generate the GST fusion construct, the insert from the His-Tag fusion construct was cut out by BamHI/EcoRI and subcloned into BamHI/EcoRI cut pGEX-5X-2 (Amersham Pharmacia Biotech). The fusion protein constructs were then transformed into E. coli strain BL2I(DE3) and induced with IPTG for protein production.

To generate the Myc-His tagged hGrpL expression plasmid the coding region of hGrpL was amplified using the following primer pairs: forward primer, 5'-GCG GAG ATG TGG AAG CTG TTG CCA AGT TTG ATT TCA C; reverse primer, 5'-GGA GGA TGG AAG CTG TTG CCA AGT TTG ATT TCA C. Underlined nucleotides represent XhoI and EcoRI sites in the forward and reverse primers, respectively. The PCR product was cloned into the pcDNA3.1 (+)/My-His A vector (Invitrogen), in-frame with the c-Myc epitope and poly-His tags. The hGrpL-Myc-His cassette was then excised with BamHI and PmeI and subcloned into the pEF expression vector (provided by Dr. G. Koretzky) at the BamHI and filled-in Xbal sites (pEF-GrpL). The pEF-SLP-76 and the NF-AT luciferase reporter (NF-AT Luc) constructs were also gifts from Dr. G. Koretzky.

Northern blot analysis and RT-PCR. To detect GrpL message in cell lines, ~30 μg of RNA were resolved on 1.2% morpholine propane sulfonic acid (MOPS)/formaldehyde agarose gels, transferred onto GeneScreen Plus membranes (NEN™ Life Science Products, Boston, MA), and fixed by UV cross-linking. Multiple Northern blots were obtained from Clontech. Northern blots were prehybridized and hybridized to the full-length GrpL cDNA or β-actin probes labeled with α-[32P]dCTP (NEN™ Life Science Products) according to the manufacturer's instructions. RT-PCR was performed using the Titan™ One Tube RT-PCR System (Boehringer Mannheim). The reaction was carried out using 1 μl of total RNA and 20 nM forward and reverse primers. Primers used were: forward primer, 5'-TGG AAG ATG ATT TTG AGT ATG TTA C; reverse primer, 5'-CTT CCT CGG TGG TCT GTC TTG CCC AGT TTG AGT ATG TTA C; reverse primer, 5'-CTT CCT CGG TGG TCT GTC TTG CCC AGT TTG AGT ATG TTA C.

Materials and Methods

Cells and Antibodies. Various cell lines were maintained in standard culture media supplemented with l-glutamine, nonessential amino acids, sodium pyruvate, penicillin/streptomycin, and fetal bovine serum. A rabbit anti-human (h)GrpL serum was generated in this study against a His-tag fusion protein containing the SH2 domain of hGrpL as described below. Antiphosphotyrosine 4G10 and anti-LAT/pp36/38 were purchased from U pstate Biotechnology, Inc. Anti-SLP-76, anti-Grb2, anti-SH2 domain-containing protein tyrosine phosphatase 2 (SHP-2), anti-SoS1, and anti-SoS2 were purchased from Santa Cruz Biotechnology, 64.1 (anti-CD3) was provided by Dr. John H ansen (Fred Hutchinson Cancer Research Center, Seattle, WA), C305 (anti-TCR) was provided by Dr. G. Koretzky (University of Iowa, Iowa City, IA), and OKT3 (anti-CD3) was obtained from the American Type Culture Collection.

Isolation of the Full-Length cDNA Clones Encoding Human and Murine GrpL. A full-length cDNA encoding hGrpL was identified through random sequencing of clones from a library prepared from the acute myelogenous leukemia cell line KG-1a. Overlapping partial cDNA clones encoding murine (m)GrpL were isolated by low stringency reverse transcriptase (RT)-PCR using multiple hGrpL-specific primer sets and murine splenic total RNA as the template. RT-PCR at low annealing temperature was performed according to the Titan™ One Tube RT-PCR System protocol (Boehringer Mannheim Inc.). PCR products were resolved on 1.5% agarose gels. cDNA bands corresponding in size to the estimated lengths of hGrpL were excised from the gel and eluted using the QIAEX II Gel Extraction Kit (QIAGEN Inc.) according to the suppliers protocol. Isolated cDNAs were then ligated into a T/A cloning vector (Invitrogen) and transformed into competent E. coli (Invitrogen). Recombinant clones containing mGrpL inserts were selected by low stringency hybridization to a hGrpL cDNA probe and then sequenced. This allowed for the identification of primer sets specific for mGrpL for the isolation of cDNA clones encoding both the 5' and 3' ends of mGrpL by rapid amplification of cDNA ends. The gene-specific primers chosen were: 5'-cDNA end, 5'-CTG ATG TCG TGG GTG CCA ACT GGA GAC ATT CTG; 3'-cDNA end, 5'-GCA TCA GCG TGG CGT CTC CAC TAC TGC C. The Marathon-Ready™ cDNA kit (Clontech Laboratory Inc.) was used for RACE PCR and performed as recommended by the supplier. The resulting PCR products were cloned into T/A cloning vectors (Invitrogen) and sequenced.

Plasmid Constructs. To generate the GrpL SH2 His-tag fusion protein, PCR was used to amplify a piece of cDNA encoding the SH2 domains of GrpL using the full-length GrpL cDNA as template and the following primer pairs forward primer, 5'-GGG ATG TGG ACC TCC CAG TTT CCC AAA TGG TTT CAC; reverse primer, 5'-GGG GGG AAT TCC TCT TGT GTC TCT TCT AAG GAA GAT CTC G. Underlined nucleotides represent BamHI and EcoRI sites in the forward and reverse primers, respectively. PCR products were cut with these enzymes and ligated into BamHI/EcoRI sites in the forward and reverse vectors, respectively. PCR products were cut with these enzymes and ligated into BamHI/EcoRI sites in the forward and reverse vectors, respectively. The PCR product was cloned into the pcDNA3.1(+) (Invitrogen), in-frame with the c-Myc epitope and poly-His tags. The hGrpL-Myc-His cassette was then excised with BamHI and PmeI and subcloned into the pEF expression vector (provided by Dr. G. Koretzky) at the BamHI and filled-in Xbal sites (pEF-GrpL). The pEF-SLP-76 and the NF-AT luciferase reporter (NF-AT Luc) constructs were also gifts from Dr. G. Koretzky.

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This primer set amplifies a 452-bp fragment containing the NH₂-terminal SH3 and the SH2 domains from cells expressing GrpL message. The amount of total RNA used was monitored by amplification of a 1-kb fragment encoding G3DPH using the following primer set: forward primer, 5'-TGA AGG TCG GAG TCA ACG GAT TTT GGT; reverse primer, 5'-CAT GTG GGC CAT GAG GTC CAC CAC. Cycling parameters PCR were programmed according to the instructions provided with the Titan™ system (Boehringer Mannheim).

**T Cell Stimulation, Immunoprecipitation, and Immunoblot Analysis.** Jurkat T cells were pelleted and resuspended at 5–10 × 10⁶/ml in medium. Cells were allowed to equilibrate at 37°C for 15 min and then stimulated with either 5 or 10 μg/ml of anti-CD3 or 2.5 mM of H₂O₂ plus 250 μM of sodium orthovanadate (referred to as peroxvanadate). Either class-matched antibodies or medium only was used as control stimulus. Stimulation was terminated by dilution of cell suspensions into >10 vol of ice-cold PBS. Cells were pelleted and lysed in a buffer containing 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 0.5% N-P-40, protease inhibitors (2 mM PM SF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 μg/ml pepstatin), and phosphatase inhibitors (10 mM NaF, 1 mM Na₃VO₄, and 5 mM Na₂P₂O₇). Lysates were clarified by centrifugation before immunoprecipitation. Immunoprecipitation, SDS-PAGE, and Western blot analysis were conducted as described (35). Binding of primary antibodies to blots was detected with horseradish peroxidase–conjugated secondary antibodies or streptavidin (Jackson ImmunoResearch Labs) and an enhanced chemiluminescence kit (American Pharmaceuticals).

**T Cell Transfection and Luciferase Assays.** Jurkat cells in log-phase were washed with serum-free RPMI 1640 once and electroporated in triplicate with a total of 60 μg of plasmid at 240 V, 960 μF using a Gene Pulser (Bio-Rad) at 10⁶ cells/400 μl reporter lysate (Promega) added to the mix. luciferase activity was quantified by adding 10 μl of the lysate to 50 μl of luciferase assay substrate (Promega) and immediately measured with a GenProbe Leader™ 1 luminometer (Wallac Inc.). To normalize for variations in transfection efficiency, luciferase activities in different transfection conditions were expressed as a percentage of maximal NF-AT responses determined by treating cells with 50 ng/ml PMA and 1 μM ionomycin as previously described (18, 20).

**Results**

Identification of GrpL, a New Member of the Grb Family of Adapter Proteins. Through random sequencing of cDNA clones in a library prepared from the acute myelogenous leukemia cell line KG-1a, we identified a cDNA encoding a 330-amino acid polypeptide with a predicted molecular weight of ~37 kDa. The linear amino acid sequence of this polypeptide organizes into an NH₂-terminal SH3 domain followed by an SH2 domain, a proline-rich region, and finally a COOH-terminal SH3 domain (Fig. 1 A). A comparison to other known proteins available in current sequence data bases revealed that this protein is most similar to the adaptor proteins, Grb2 and Grap (Fig. 1 B and Table 1). Based on this similarity, we have designated this molecule, GrpL, for Grb2-related protein of the lymphoid system (see below). Using RT-PCR and a combination of 5'- and 3'-RACE (Materials and Methods), we also isolated the full-length cDNA encoding the murine homologue of GrpL. Human and murine GrpL are highly conserved proteins sharing >90% identity in the SH3 and SH2 domains (Table 1). The least conserved area is the proline-rich region with ~74% identity (Table 1). Amino acid sequence alignment between hGrpL, Grb2, and Grap revealed that hGrpL is 38–56% identical to Grb2 and Grap in the SH3 and SH2 domains (Fig. 1 B, Table 1). This conservation is slightly less than that between Grb2 and Grap (49–68% reference 33).

The biggest difference between GrpL and Grb2 or Grap is the presence of a proline-rich region between the SH2 and COOH-terminal SH3 domain. This proline-rich region could potentially bind by SH3 domain–containing proteins found in other adaptor molecules (36, 37). Taken together, the amino acid composition of GrpL suggests that it may interact with different proteins in lymphocytes when compared with Grb2 or Grap.

Transcripts of GrpL Are Selectively Expressed in Hematopoietic Tissues and in B and T Lymphocytes. The transcripts for Grb2 are expressed ubiquitously in mammalian species (38), whereas Grap mRNA is expressed at considerably higher levels in lymphoid tissues than in other tissues (33, 34). Northern blot analysis of mRNA isolated from different human tissues demonstrated that the transcripts for GrpL are relatively restricted to hematopoietic tissues. Thus, transcripts of 1.4 kb and 3.5 kb were detected in lymph node, bone marrow, spleen, thymus, and peripheral blood lymphocytes, as well as weakly in testis, but not in stomach, thyroid, spinal cord, trachea, adrenal gland, prostate, ovary, or small intestine (Fig. 2 A). Probing total RNA from a panel of cell lines by Northern blot analysis and RT-PCR further confirmed the restricted expression pattern of GrpL (Fig. 2, B and C). GrpL transcripts were detected in the myeloid and erythroid progenitor cell lines KG-1a and K562, respectively, but not in the myeloid cell line HL60. In T cells, GrpL is expressed in the Jurkat, Molt-4, HSB-2, CEM, and HP-ALL lines, but not in the HuT78 line. Interestingly, all of the B cell lines examined, GrpL transcripts were only found in EBV-transformed lymphoblastoid cell lines including T5-1, HCLL7678, CESS, F4, FBM 2-4, and IM 9. Other B cell lines, including the Burkitt’s lymphomas (Daudi, BJAB, and Ramos), the pre-B cell lines (REH, NALM-6, and BLIN1), the sIgM-expressing lymphomas (Daudi, BJAB, and Ramos), the pre-B cell lines (REH, NALM-6, and BLIN1), the sIgM-expressing lymphomas (Daudi, BJAB, and Ramos), and the myeloma cell lines RPMI-8226, did not express any GrpL transcripts. Results from the above experiments show that GrpL expression is highly regulated and restricted to subsets within hematopoietic lineages.

GrpL Associates with SLP-76 in T Cells. To characterize the GrpL protein and the proteins with which it interacts,
we generated a rabbit hetero-antiserum specific for GrpL by immunizing with a His-Tag GrpL-2 fusion protein. This antiserum can specifically immunoprecipitate from Jurkat T cells two proteins ~38 and 40 kD in size (Fig. 3 A). Both proteins were detectable by Western blot analysis of either GrpL immunoprecipitates (IPs) or whole cell lysates. This antiserum does not cross-react with Grb2, which runs at a lower molecular weight on gels (Fig. 3 A and see Fig. 5), and by all criteria it appears to be specific for GrpL.

To detect proteins potentially capable of interacting with GrpL, we pharmacologically stimulated Jurkat cells with pervanadate, immunoprecipitated GrpL, and then Western blotted with either antiphosphotyrosine or anti-GrpL sera (Fig. 3 B). 2-5 min after stimulation, a major tyrosine phosphorylated protein ~40 kD in size and a minor 38-kD phosphoprotein were detectable in anti-GrpL but not control serum IPs. This doublet most likely corresponds to GrpL itself, which migrates to the same position on gels (Fig. 3, A and B); it is possible that these bands represent differentially phosphorylated forms of GrpL. Compared with pervanadate, TCR ligation induced only weak tyrosine phosphorylation of GrpL (data not shown).
In addition to the 38–40-kD doublet, a second major tyrosine phosphorylated protein ~68–70 kD in size was also detected in the GrpL IP after pervanadate treatment (Fig. 3, B and C). In T cells, substrates for protein tyrosine kinases in this size range include SLP-76, SHP-2, Syk, and ZAP-70. Indeed, in the GrpL IP obtained from both unstimulated and pervanadate-stimulated Jurkat cells, we were able to detect SLP-76 (Fig. 3 C) but not ZAP-70 (data not shown). However, the amount of SLP-76 associating with GrpL remained relatively unchanged after stimulation. Moreover, the SLP-76 molecule communoprecipitated with GrpL before stimulation showed an almost undetectable level of tyrosine phosphorylation (Fig. 3 C). These results are consistent with the idea that GrpL can interact with SLP-76 independently of tyrosine phosphorylation as has been suggested for Grb2 and SLP-76 (21, 27). The increased signal of the 68–70-kD molecule communoprecipitated with GrpL after stimulation may be a consequence of increased tyrosine phosphorylation of SLP-76 and/or the recruitment of additional tyrosine phosphorylated proteins of the same size to GrpL. In the SLP-76 IP obtained from pervanadate-stimulated Jurkat cells, we could also detect tyrosine-phosphorylated protein at 38–40, 50–55, and 110 kD (Fig. 3 C).

GrpL and Grb2 Interact with Different Molecules. To examine the possibility of distinct functional roles played by different Grb2-like molecules in T cells, we asked whether GrpL and Grb2 interact with different proteins. We immunoprecipitated either GrpL or Grb2 from Jurkat cells and probed for associating proteins. SLP-76 was detected in GrpL IPs (Fig. 4 A), as also shown in Fig. 3 C. However, we could not detect any SLP-76 associating with Grb2. Consistent with this was the presence of GrpL but not Grb2 in the SLP-76 IPs (Fig. 4 A). On the other hand, we were able to detect Sos1 and Sos2, GEFs of Ras, in Grb2 IPs (Fig. 4 B), as has been reported in other cell systems. Interestingly, no detectable Sos1 or Sos2 was found to be communoprecipitated with GrpL (Fig. 4 B). These results suggest that in Jurkat T cells, GrpL preferentially complexes with SLP-76, whereas Grb2 preferentially complexes with Sos1 or Sos2.

Since both Grb2 (10–14) and Grap (33, 34) can associate with tyrosine-phosphorylated LAT/pp36/38 upon TCR ligation, we asked whether GrpL could also associate with tyrosine-phosphorylated LAT/pp36/38. Jurkat cells were stimulated by anti-CD3, and LAT/pp36/38 was immunoprecipitated from NP-40 Jurkat cell lysates at various time points after stimulation. As reported before (24), tyrosine-phosphorylated LAT/pp36/38 was not detected in the GrpL IPs (Fig. 4 A). We were able to detect SLP-76 in the GrpL IPs (Fig. 4 A), as also shown in Fig. 3 C. However, we could not detect any SLP-76 associating with Grb2. Consistent with this was the presence of GrpL but not Grb2 in the SLP-76 IPs (Fig. 4 A). On the other hand, we were able to detect Sos1 and Sos2, GEFs of Ras, in Grb2 IPs (Fig. 4 B), as has been reported in other cell systems. Interestingly, no detectable Sos1 or Sos2 was found to be communoprecipitated with GrpL (Fig. 4 B). These results suggest that in Jurkat T cells, GrpL preferentially complexes with SLP-76, whereas Grb2 preferentially complexes with Sos1 or Sos2.

**Table I.** Amino acid sequence comparison among hGrpL, mGrlP, Grb2, and Grap

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<tr>
<td>hGrpL/mGrpL</td>
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<td>93</td>
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<tr>
<td>hGrpL/Grb2</td>
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Numbers represent the percentage of identity between the different domains of the two proteins listed on the lefthand column.
Figure 4. GrpL and Grb2 interact with different effector molecules. (A) N P-40 lysates prepared from Jurkat cells were immunoprecipitated with anti–SLP-76 (αSLP-76), anti-GrpL (αGrpL), or normal goat or rabbit control Ig. IPs were resolved on SDS-PAGE and immunoblotted with antisera as indicated on the left. (B) N P-40 lysates were preincubated with anti–GrpL or preimmune serum and immunoblotted with the antiphosphotyrosine antibody 4G10 (αPY). The amounts of GrpL in different lanes were monitored by immunoblotting with anti-GrpL (αGrpL). The open arrow indicates a predominant tyrosine-phosphorylated protein coimmunoprecipitating with GrpL, and the closed arrow indicates the mobility of GrpL. (C) N P-40 lysates prepared from unstimulated (-) or pervanadate-stimulated (+) Jurkat cells were immunoprecipitated with anti-SLP-76 (αSLP-76), anti-GrpL (αGrpL), or normal goat or rabbit control Ig. IPs were resolved on SDS-PAGE and immunoblotted with either antiphosphotyrosine or anti-SLP-76. The arrows indicate phosphoproteins migrating at the sizes of SLP-76 and GrpL.

Figure 3. Biochemical characterization of GrpL. (A) Jurkat cell lysates were immunoprecipitated with an antiserum against GrpL, generated by immunizing rabbits with the GST/GrpLSH2 fusion protein or preimmune serum. IPs were resolved along with whole cell lysate on reducing SDS-PAGE and immunoblotted with the same anti-GrpL serum. (B) Jurkat cells were stimulated for either 2 or 5 min with pervanadate as described in Materials and Methods. N P-40 lysates were immunoprecipitated with anti-GrpL or preimmune serum and immunoblotted with the antiphosphotyrosine antibody 4G10 (αPY). The amounts of GrpL in different lanes were monitored by immunoblotting with anti-GrpL (αGrpL). The open arrow indicates a predominant tyrosine-phosphorylated protein coimmunoprecipitating with GrpL, and the closed arrow indicates the mobility of GrpL. (C) NP-40 lysates prepared from unstimulated (-) or pervanadate-stimulated (+) Jurkat cells were immunoprecipitated with anti–SLP-76 (αSLP-76), anti-GrpL (αGrpL), or normal goat or rabbit control Ig. IPs were resolved on SDS-PAGE and immunoblotted with either antiphosphotyrosine or anti-SLP-76. The arrows indicate phosphoproteins migrating at the sizes of SLP-76 and GrpL.

phosphorylation of LAT/pp36/38 increased dramatically after TCR ligation, and started to decline after 3 min of stimulation. This was correlated with the appearance of Grb2 in the LAT/pp36/38 IPs (Fig. 5). However, although the levels of GrpL proteins in N P-40 Jurkat cell lysates remained unchanged throughout stimulation, we consistently did not detect any GrpL associating with LAT/pp36/38 in these lysates (Fig. 5).

GrpL Regulates NF-AT Activation in T Cells. Since SLP-76 has been shown to regulate NF-AT activation in T cells (18, 20, 39), we investigated whether GrpL could play a role in this pathway. NF-AT activation was monitored by the expression of a NF-AT luciferase reporter construct in Jurkat cells. As described before, overexpression of SLP-76 by cotransfection of a SLP-76 expression vector augmented the anti-CD3-induced NF-AT activation (18, 20, 39). Overexpression of GrpL alone consistently augmented anti-CD3-induced NF-AT activation (Fig. 6). Moreover, an additive effect between GrpL and SLP-76 on NF-AT activation was observed when both were overexpressed in Jurkat cells.

The SH2 Domain of GrpL Recognizes Tyrosine-phosphorylated Protein Tyrosine Phosphatase SHP-2 In Vitro. Since various SH2 domains can interact with a wide spectrum of tyrosine-phosphorylated targets, we tested to which tyrosine-phosphorylated protein(s) the SH2 domain of GrpL could bind. When the GST/GrpLSH2 fusion protein was used to probe pervanadate-stimulated Jurkat cells, tyrosine-phosphorylated proteins that migrated at 36, 69, and 71 kD

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could be readily detected (Fig. 7 A). In some experiments, we also detected 95- and 130-kD tyrosine-phosphorylated proteins. The GST/GrpLSH2 fusion protein also precipitated a similar pattern from lysates prepared from anti-CD3–stimulated Jurkat cells (Fig. 7 A). By analogy to Grb2 (24), these proteins could be LAT, ZAP-70, SHP-2, Vav, and perhaps c-Cbl. As shown in Fig. 7 B, we were able to confirm that SHP-2 was among the phosphoproteins precipitated by the GST/GrpLSH2 fusion protein from pervanadate-activated Jurkat cells. Although tyrosine-phosphorylated SLP-76 also migrates at ~70 kD, we consistently did not detect any SLP-76 precipitated by GST/GrpL.

Discussion

Critical functions are played by a variety of adaptor molecules in linking receptor proximal signal transduction events such as activation of receptor-associated kinases to downstream effector molecules. In this report, we describe the identification and characterization of a new member of the Grb family of adaptor proteins. GrpL shares similar structural organization as Grb2 and Grap. A distinguishing feature of GrpL not found in Grb2 or Grap is the presence of a 100-amino acid stretch connecting its SH2 and SH3C domains. Although this region is the least conserved between mGrpL and hGrpL, it does contain multiple proline and glutamine residues at conserved positions (Fig. 1 and Table I). Thus, two PxxP motifs are found in both hGrpL and mGrpL. In hGrpL, they are P191TLP and P202QPP; in mGrpL, they are P190LGP and P210QPP. These polyproline motifs conform to the consensus sequence recognized by different SH3 domains (36, 37). Accordingly, it is likely that the proline residues in GrpL do constitute a proline-rich domain capable of interacting with as yet to be defined SH3-containing proteins.

In contrast to the broadly expression Grb2, GrpL expression is restricted to hematopoietic cells (Fig. 2). In this regard, GrpL is similar to Grap that transcripts for both are found at much higher levels in hematopoietic tissues (33, 34). Although Grap is preferentially expressed in blood cells, little is known about its expression in different stages of T and B cell maturation or in different lymphocyte subsets. GrpL transcripts are expressed at lower levels in the bone marrow than in the secondary lymphoid organs. Importantly, the highest levels of GrpL transcripts were detected in the thymus (Fig. 2 A), suggesting that GrpL may play a crucial role in regulating early T cell development.
date-stimulated Jurkat cells were immunoblotted with anti–SLP-76. GrpL precipitates. (B) Replicate precipitates obtained from pervanadate major tyrosine-phosphorylated proteins detected in the GST/munoblotted with the antiphosphotyrosine antibody 4G10. Arrows indicate control IgG or medium alone. NP-40 lysates were precipitated with either GST or GST/GrpLSH2, resolved on reducing SDS-PAGE, and immunoblotted with the anti-phosphotyrosine antibody 4G10. Arrows indicated major tyrosine-phosphorylated proteins detected in the GST/GrpLSH2 precipitates. (B) Replicate precipitates obtained from pervanadate-stimulated Jurkat precipitates were immunoblot with anti-SLP-76.

in germinal center B cells (Solow, S.A., A.J. Marshall, T.J. Yun, M.K. Ewings, and E.A. Clark, manuscript in preparation). To elucidate the signals involved in switching the expression of GrpL on and off will provide further insights into the functions of GrpL and its associating proteins at different stages of lymphocyte development.

We have identified one of the GrpL-interacting proteins to be SLP-76 (Figs. 3 and 4). Interaction between GrpL and SLP-76 appears to be constitutive, since it could be detected in both stimulated and unstimulated T cells (Figs. 3 and 4 A). Accordingly, this interaction may not require the tyrosine phosphorylation of either SLP-76 or GrpL. In support of this, results presented in Fig. 3 C show that the amount of SLP-76 communoprecipitating with GrpL remained relatively constant, although SLP-76 became heavily tyrosine-phosphorylated after pervanadate treatment. Hence, our data argue against the possibility that the SH2 domain of GrpL mediates its interaction with SLP-76. Grb2 has been reported to be an adaptor molecule capable of associating with SLP-76 (24). In these studies, GST fusion proteins containing Grb2 or Grb2 SH3 domains recognized SLP-76 from lysates of resting or activated T cells independent of its tyrosine-phosphorylation (21, 27). Using deletional mutants of SLP-76, the Grb2 binding site was subsequently mapped to the central proline-rich region of SLP-76 from amino acid residues 224–244 (21). These results implicate the SH3 domain of Grb2 to be the primary mediator for interaction. Based on the similar structure between GrpL and Grb2, and the phosphorylation-independent interaction between GrpL and SLP-76, it is likely that the SH3 domain(s) of GrpL also mediates its interaction with SLP-76. Experiments are currently in progress to examine this possibility.

Several recent studies have provided strong evidence for an essential function served by SLP-76 in T lineage cells. Biochemical experiments show that SLP-76 can interact with both positive and negative regulators. TCR ligation induces ZAP-70 to phosphorylate SLP-76 at three NH2-terminal tyrosine residues (20, 30, 39). Once phosphorylated on its NH2-terminal tyrosine residues, in particular Y113 and Y128, SLP-76 can be bound by the SH2 domain of Vav (18, 19). Overexpression of SLP-76 augments IL-2 promoter activity (21), and SLP-76 also acts synergistically with Vav to augment IL-2 promoter activity (18). Moreover, the biological activity of SLP-76 is absolutely dependent on an intact proline-rich region, and hence, an interaction with adaptor proteins containing SH3 domain(s) (21). On the other hand, the COOH-terminal SH2 domain of SLP-76 binds to a 130-kD tyrosine-phosphorylated protein (40, 41). This protein has been cloned, designated to be SLAP-130/Fyb, and may be involved in downregulating the activating function of SLP-76 on the IL-2 promoter (41). The most compelling evidence to argue for an indispensable in vivo function of SLP-76 in T lineage cell is provided by the recent gene targeting experiments. SLP-76−/− mice are characterized by an early arrest in T cell development. CD4+/CD8+, CD4+, and CD8+ thymocyte populations are completely absent from thymuses of SLP-76 knockout mice, resulting in a corresponding lack of peripheral T cells (22, 23). In the SLP-76−/− mice, TCR-β chain genes appear to undergo normal rearrangement and exogenous anti-CD3 fails to drive the generation of double positive cells. Therefore, it is proposed that the absence of SLP-76 may render the pre-TCR complex fail to deliver maturational signals to developing T cells (22, 23).

Our observation that GrpL associates with SLP-76 in vivo suggests GrpL plays a pivotal role in TCR-mediated signal transduction cascades. Studies from different laboratories have convincingly demonstrated binding of recombinant Grb2 or Grb2 SH3 domains to SLP-76 both in cell lysates and on Western blots (9, 21, 27), but the presence of SLP-76 in Grb2 IPs and vice versa have not been reported thus far. Our data argue that SLP-76 is more likely to associate with GrpL than Grb2 under physiological conditions in T cells (Fig. 4). This GrpL/SLP-76 association may regulate signaling pathway(s) involving SLP-76 in T cells. One downstream target for SLP-76 is NF-AT. SLP-76 and Vav can synergistically enhance anti-CD3-induced NF-AT activation in T cells (18). Results in Fig. 6 demonstrate that overexpression of GrpL alone is sufficient to enhance the

Figure 7. The SH2 domain of GrpL interacts with SHP-2. (A) Jurkat T cells were stimulated with 10 μg/ml of anti-CD3 (αCD3) or pervanadate for 5 min. Control cells were treated with either isotype-matched control IgG or medium alone. NP-40 lysates were precipitated with either GST or GST/GrpLSH2, resolved on reducing SDS-PAGE, and immunoblotted with the antiphosphotyrosine antibody 4G10. Arrows indicated major tyrosine-phosphorylated proteins detected in the GST/GrpLSH2 precipitates. (B) Replicate precipitates obtained from pervanadate-stimulated Jurkat cells were immunoblotted with anti-SHP-2.
Hence, after TCR ligation, the interaction between Grb2 activation and tyrosine phosphorylation of SHP-2 (Fig. 7). Binding to SHP-2. This binding is dependent on T cell activation. Similar to Grb2, the SH2 domain of GrpL mediates activity inhibiting the TCR-induced MAPK pathway activation. Thus, a multimeric complex involving Grb2, SHP-2 may also be a positive regulator of TCR-mediated signaling. For example, SHP-2 has been shown to be required for coupling receptors to the activation of the MAPK pathway (47, 48). Nevertheless, a recent report suggests that GrpL can bind to SLP-76 and together with SLP-76 can promote the activation of NF-AT in T cells. We have recently identified another adaptor protein, BAM-32, which unlike GrpL (Fig. 6 and reference 56), SLP-76 (18-21), or the M-CSF receptor, c-fms (54), or with c-kit (55). In agreement with our results, Liu et al. (56) independently found that GrpL can bind to SLP-76 and together with SLP-76 can promote the activation of NF-AT in T cells. We have recently identified another adaptor protein, BAM-32, which unlike GrpL (Fig. 6 and reference 56), SLP-76 (18-21), or the B cell adaptor BLNK (57), inhibits the activation NF-AT (Marshall, A.J., H. Niiro, T.J. Yun, and E.A. Clark, manuscript in preparation). Thus, antigen receptor-induced activation of NF-AT in lymphocytes is carefully regulated by adaptor proteins.

Figure 8. A model for how GrpL may function in TCR-mediated signal transduction. Results from our study demonstrate that GrpL and Grb2 may differentially associate with SLP-76, LAT/pp36/p38, and Sos1/2, respectively. The preferential interaction between GrpL and SLP-76 suggests that GrpL may play a role in the regulation of NF-AT pathway during TCR-mediated T cell activation.
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