The Role of a Lymphoid-restricted, Grb2-like SH3-SH2-SH3 Protein in T Cell Receptor Signaling*

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We have characterized an SH3-SH2-SH3 linker protein that is prominently expressed in lymphoid tissues. This protein has 58% sequence identity to Grb2. An identical protein called Grap has been found in hematopoietic cells. In Jurkat cells, T cell receptor activation leads to the association of Grap with phosphoproteins p36/38 and, to a lesser degree, Shc. This interaction is mediated by the Grap SH2 domain, which has similar binding specificity to the Grb2 SH2 domain. Grap also associates via its SH3 domains with Sos, the Ras guanine nucleotide exchange factor; with dynamin, a GTPase involved in membrane protein trafficking; and with Sam68, a nuclear RNA-binding protein that serves as a substrate of the association of Grap with phosphoproteins p36/38, Shc, Sos, and dynamin. Sam68 binding is constitutive. Phospholipase C-γ1 and Fyn are also found in activated Grap signaling complexes, although these interactions may not be direct. We conclude that Grap is a prominent component of lymphocyte receptor signaling. Based on the known functions of bound effector molecules, Grap-mediated responses to antigen challenge may include endocytosis of the T cell receptor, cellular proliferation, and regulated entry into the cell cycle.

Activation of resting T cells through the T cell antigen receptor triggers a cascade of intracellular biochemical events that lead to lymphocyte differentiation and proliferation (1–3). Although components of the T cell receptor and associated CD3 and ζ chains lack intrinsic kinase activity, tyrosine phosphorylations are among the earliest biochemical events detected after activation (4). The phosphorylation of CD3/ζ and other cellular proteins is essential in T cell activation (5, 6). Two types of cytoplasmic tyrosine kinases are known to catalyze these phosphorylations. The Src family kinases Lck and Fyn associate with the cytoplasmic portions of the CD3/ζ and CD4/CD8 co-receptors, respectively, and phosphorylate regularly spaced, paired tyrosine residues within ITAM motifs of the ζ and CD3 cytoplasmic tails. The ZAP-70 tyrosine kinase is recruited to the activated complex by binding phosphorylated ITAM motifs via its tandem SH2 domains. Sequential or combinatorial activation of the ZAP-70 and Src family kinases leads to phosphorylation of numerous additional proteins, including Shc, p36/38, Lnk, SLP-76, phospholipase C-γ1 (PLC-γ1), 1 and the protooncoproteins c-Cbl and Vav. Many of these substrates contain SH2 domains. Several additional SH2 domain proteins that are not tyrosine-phosphorylated, such as Grb2 and phosphatidylinositol (PI) 3-kinase, are also recruited to the activated receptor. SH2 domain-phosphoprotein interactions, therefore, provide a mechanism for the assembly of protein complexes that participate in propagating antigen receptor signals to downstream cellular targets. Similar events follow antigen receptor activation in B lymphocytes.

Many critical proteins involved in lymphocyte signal transduction are ubiquitously expressed, including enzymes in phosphoinositide pathways such as PLC-γ1 and PI 3-kinase and Ras effectors like Grb2 and Sos. In contrast, the expression of signaling proteins such as Lek, ZAP-70, SHP1, Vav, SLP-76, Lnk, and the T cell receptor/CD3 components is much more restricted. Recent advances in cloning and gene sequencing technology, particularly random genome sequencing efforts, allow the identification of additional cell type-specific proteins with potential roles in cell signaling. Based on sequence homology, a cDNA clone encoding a new adaptor protein was identified in the Human Genome Sciences expressed sequence tag (EST) data base (7). This protein has an SH3-SH2-SH3 domain architecture and 58% sequence identity to Grb2. Northern analyses of mRNA derived from multiple human tissues show restricted expression in spleen, thymus, and peripheral blood leukocytes. Using a specific antibody, we detected the protein in isolated peripheral blood lymphocytes and in all established T and B cell lines tested. These findings suggest potential functions for this protein in T cell signaling. Independently, an identical protein, designated Grap (Grb2-like accessory protein), was identified in hematopoietic cells and found to have potential functions downstream from erythropoietin and stem cell receptors (8).

A large number of studies have established an important role for Grb2 in linking tyrosine kinase receptors to downstream effector pathways. For example, the SH2 domain of Grb2 binds activated growth factor receptors either directly or indirectly

1 The abbreviations used are: PLC, phospholipase C; PI, phosphatidylinositol; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pairs; IL, interleukin; NF-AT, nuclear factor of activated T cells.

2 Sequence identified by Drs. Damien Dunnington and Mark Hurle, SmithKline Beecham Pharmaceuticals.
Triton X-100 (1.8–2.0%) was added to lysates containing N-laurylsarcosine. The proteins were adsorbed to immobilized glutathione agarose (Molecular Probes), and the columns were washed with buffer W (25 mM Tris/HC1, pH 8.0, 100 mM NaCl, 1.0 mM EDTA, 1.0 mM diithiothreitol). The protein-tagged mSos1 fragment was expressed in E. coli strain BL21(DE3)(Novagen). After induction with 0.4 mM isopropyl-\(\beta\)-D-thiogalactopyranoside, the bacteria were disrupted by sonication, and the soluble protein was affinity-purified using a Ni\(^2+\) chelating column (Novagen) as described by the supplier. To generate biotinylated probes, eluted proteins (2–4 mg/ml) were dialyzed against 100 mM sodium borate, pH 8.8, treated with biotinamidocapro-N-hydroxy succinimidyl ester (Sigma) for 4 h at 22°C (28), and dialyzed against buffer W. Protein purity and concentrations were assessed by Coomassie staining following separation by SDS-PAGE.

**Peptides and Antibodies—**Peptides synthesized using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry and high pressure liquid chromatography-purified (27) are numbered by position within the corresponding protein: Lnk-pY299, DNQpYTPLSQL; Shc-pY317, PpspYVpQNL; Shc-pY317/N318A, PpspVYAVpQL; BCR-pY177, KFP-pYVNYEF; Grap-199 RSGCVRGFPSSVYQVPHL. For precipitation experiments, peptides were coupled to Affi-Gel 10 (Bio-Rad). Peptides (2–10 mg/ml) dissolved in Me\(_2\)SO containing 25 mM N-ethylmaleimide were incubated with equal volumes of washed Affi-Gel 10 for 16 h at 22°C. Excess reagents were removed, and unreacted sites on the resin were blocked by treatment with ethanolamine. The resin was then incubated with Grap. Grap was prepared by incubation of rabbits with peptide Grap-199 coupled to maleimide-activated keyhole limpet hemocyanin (Pierce). Grap-specific antibodies were affinity-purified by passage of the antisera over immobilized Grap-199, extensive washing of the beads with phosphate-buffered saline, and elution with 0.1 M HCl. Additional antibodies used in these studies include SFV-Tb (anti-CD3e) (28) and anti-Cbl (24). Rabbit anti-mouse IgG (6170-01) was from Southern Biotechnology; anti-dynamin (D25520), anti-Shc (S14630), anti-Sos1 (15520), and agarose-coupled anti-phosphotyrosine (P11821) were from Transduction Laboratories; anti-Grb2 (225), anti-PLC-\(\gamma\) (426), anti-Sam68 (333), and anti-SOS1/2 (259) were from Santa Cruz Biotechnology; and anti-p55 (05-212) and anti-phosphotyrosine 4G10 were from UBI. The anti-Myc epitope antibody was purified with immobilized protein G from culture supernatants of the hybridoma 9E10 kindly provided by J. M. Bishop (University of California, San Francisco) (29).

**Cell Culture and T Cell Activation—**Cells were cultured in RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum (HyClone), 2 mM L-glutamine (Life Technologies, Inc.), 50 \(\mu\)g/mL 2-mercaptoethanol, and streptomycin/penicillin (Life Technologies), in the presence of 2% fetal bovine serum (FBS). Normal peripheral blood T cells (phytohemagglutinin blast), obtained by stimulation of human buffy coat-derived mononuclear cells with phytohemagglutinin (Pharmacia; 1:2000 dilution), were grown in media containing 1.5% CO\(_2\). Normal peripheral blood T cells (phytohemagglutinin blast), obtained by stimulation of human buffy coat-derived mononuclear cells with phytohemagglutinin (Pharmacia; 1:2000 dilution), were grown in media containing 1.5% CO\(_2\). 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**Fig. 1.** Grap transcripts are expressed in spleen and thymus. Membranes containing mRNA from multiple human tissues were probed as described under “Results.” The 2.1-kb Grap transcript is expressed predominantly in spleen and thymus, with less in peripheral blood leukocytes. The 3.5-kb Grb2 is present in all tissues.

**RESULTS**

**Tissue Distribution of Grap mRNA and Protein Expression**—The entire cDNA for Grap and the coding sequence for Grb2 were radiolabeled and used to probe multiple-tissue Northern blots. A 2.1-kb transcript was detected for human Grap, compared with a 3.5-kb transcript for human Grb2 (Fig. 1). Highest levels of Grap mRNA were found in spleen and thymus, with an intermediate amount in peripheral blood leukocytes. Much less of the transcript was detected in alternative tissues, suggesting that Grap expression is predominant in lymphoid tissues. Apparent expression of Grap at low levels may reflect the presence of immune cells within these tissues. For comparison, equivalent amounts of a 3.5-kb human Grb2 transcript were found in all tissues (Fig. 1), as previously reported (32).

Polyclonal antisera were generated against the C-terminal 19 residues of Grap to detect the expressed protein. The corresponding region provides useful anti-Grb2 antibodies (e.g., Santa Cruz), and the protein sequences diverge within this region. Antibodies were concentrated and nonreacting species were eliminated by immobilized Grap peptide affinity purification. GST-Grap was readily recognized by the anti-Grap antibodies in immunoblots. GST-Grb2 was recognized by commercial anti-Grb2 antibodies (Santa Cruz). There was no detectable cross-reaction (i.e. affinity-purified anti-Grap antibodies did not recognize Grb2, and vice versa) (Fig. 2). However, the Grap antibodies were not useful for immunoprecipitation, and gave high backgrounds when attempts were made to immobilize SDS-PAGE-separable cell lysates. Therefore, endogenous Grap and Grb2 proteins were quantitatively extracted from cell lysates with immobilized phosphopeptide beads. The peptide sequence was derived from the region surrounding Tyr^{377} of the breakpoint cluster region of BCR-ABL (Lys-Pro-Pro-Phes-Tyr(P)-Val-Asn-Val-Glu-Phes), which binds Grb2 and Grap with equally high affinity (Refs. 11 and 33 and this study; see below).

Proteins from lysates of several cell lines were precipitated using an excess of immobilized BCR-ABL peptide beads, separated by SDS-PAGE, and detected by immunoblotting. Cells of lymphocytic origin contained Grap, including normal peripheral T cells (phytohemagglutinin blasts) and cultured T (Jurkat) and B (Ramos, Raji, and Daudi) cell lines (Fig. 2). In contrast, all cells tested, including erythroleukemia line K562 and Va2 fibroblasts, contained Grb2. In conjunction with the Northern analyses, these results suggest a restricted role for Grap in T lymphocyte signaling, compared with a more general role for Grb2 in the signaling pathways of many cells. Because the phosphopeptide beads extracted both Grap and Grb2 quantitatively, relative amounts of the proteins could be estimated by comparing blotting intensities with various fusion protein dilutions. Grap is present in B and T lymphocytes at about 5 ng/mg total protein. Grb2 is present in the same cells at approximately 10-fold higher concentration (Fig. 2).

**p36/38 Is a Phosphoprotein Partner of Grap—**Jurkat cells were activated by anti-CD3 cross-linking to determine whether Grap is involved in T cell signaling. Phosphoproteins were immunoprecipitated with anti-phosphotyrosine antibodies and Grap was detected by immunoblotting with the anti-Grap antibody. Receptor activation led to a substantial increase in the amount of Grap that co-precipitated with phosphoproteins (Fig. 3), indicating that at endogenous levels of all cellular constituents, Grap functions in a signaling pathway downstream from the T cell receptor.

To begin to learn which cellular proteins interact with Grap, Jurkat cells were activated, and proteins were precipitated with GST-Grap and GST-Grb2 fusion proteins. Two prominent, tyrosine-phosphorylated, 36- and 38-kDa proteins co-precipitated with GST-Grap in lysates of stimulated but not unstimulated cells (Fig. 4). No other phosphoproteins were readily detected. In contrast, GST-Grb2 interacted with numerous tyrosine-phosphorylated proteins in Jurkat cell lysates, including 36- and 38-kDa proteins (Fig. 4). Previous studies suggest that in lymphocytes, Grb2 associates directly or indirectly with Shc (p52), PLC-γ1, p36/38, c-Cbl (p120), SLP-76 (p76), and ZAP-70 (p70). Several of these phosphoproteins account for additional bands present in GST-Grb2 precipitates. Whereas Grap and Grb2 precipitate equivalent amounts of p36/38, Grap does not associate substantially with the alternative Grb2-bound phosphoproteins. Based on the time course of phosphorylation (not shown) and relative mobilities by SDS-PAGE, the Grap-associated p36/38 appears to be the same protein(s) known to interact with Grb2, PLC-γ1, and PI 3-kinase p85 in activated T cells (31, 34–36).

**p36/38 Binds Grap in Cells—**Grap was expressed in Jurkat cells in order to learn more about its role in T cell receptor signaling. An anti-Myc antibody epitope was fused to the C terminus of Grap for immunoidentification. The Myc-tagged Grap was expressed transiently in an SV40 large T antigen-expressing Jurkat cell line, JMC-T (31), and after 72 h, cells were stimulated with anti-CD3 antibodies. Grap-Myc protein was immunoprecipitated from lysates using anti-Myc antibodies. Lysates from unstimulated and stimulated cells contained equivalent amounts of the protein, as detected by immunoblotting with anti-Grap antibodies (Fig. 5A). Associated proteins...
Grap associates with endogenous phosphoproteins upon T cell receptor activation. Jurkat cells were stimulated (+) or not (−) for 2 min with anti-CD3 antibody (αCD3), lysed, and immunoprecipitated with anti-phosphotyrosine antibody (αpTyr). Proteins were separated by SDS-PAGE and immunoblotted with anti-Grap antibody (αGrap). In control lane C immunoprecipitations were conducted without cell lysate.

Grap interacts with p36/38 via its SH2 domain. Jurkat cells were stimulated (+) or not (−) for 2 min with anti-CD3 antibody (αCD3), lysed, and incubated with immobilized GST-Grap or GST-Grb2 fusion proteins. GST-Grap has the wild-type sequence (3-2-3), an R86K mutation that abolishes SH2 domain binding (3-2-3*), or P49L and P208L mutations that prevent binding to both SH3 domains (3-2-3**). Potential positions of known phosphoproteins are denoted at the right (p120 Cbl, p76 SLP-76, p70 ZAP-70, p52 Shc, and p36/38).

Peptide Binding Specificity of the Grap SH2 Domain—Grb2 interacts with numerous phosphoproteins in activated T cells, while Grap binds primarily to p36/38 (Fig. 4). Phosphopeptide binding specificities of Grb2 and Grap were compared in order to test whether a restricted SH2 domain binding specificity might account for the limited number of proteins interacting with Grap. Immobilized GST-Grb2 and GST-Grap were used to precipitate p36/38 in the presence of varying concentrations of a Shc-derived phosphopeptide (Fig. 6, A and B). The Shc peptide corresponds to the sequence surrounding Tyr317 (Pro-Ser-Tyr(P)-Val-Asn-Val) and recognition motif for Grb2. This peptide blocks interactions with both proteins at equally low concentrations (ID50 = 1 μM). Findings are similar with isolated peptide binding assays. ID50 values for Grb2 and Grap binding to the Shc-pY317 peptide are 4.4 ± 0.2 μM and 1.6 ± 0.4 μM, respectively (Fig. 6, C and D). The oncoprotein BCR-ABL binds Grb2 at Tyr317 within a similar motif (33). A BCR pY177 peptide (Lys-Pro-Phe-Tyr(P)-Val-Asn-Val-Glu-Phe) binds Grb2 and Grap SH2 domains with equivalent high affinity (ID50 = 2.1 ± 0.4 μM and 0.8 ± 0.3 μM, respectively). Asparagine at the Tyr(P)-2 position is critical for binding to both proteins, since substitution with alanine substantially reduced affinity (ID50 >300 in both cases; Fig. 6, C and D), and tyrosine phosphorylation is necessary as well (data not shown). These results demonstrate that Grap and Grb2 SH2 domains have similar peptide binding specificities.
VEF (PSpYVAVQNL) SH3 domains of Grb2 in lymphocytes: the SH2 domain protein, main partners of Grap. Two phosphoproteins interact with the stoichiometry (31, 35, 36).

...for Grb2 in Jurkat cells. The mixtures were incubated with immobilized recombinant GST-Grb2 or GST-Grap; bound proteins were separated by SDS-PAGE and detected by immunoblotting with anti-phosphotyrosine antibody. C and D, binding specificities were also determined using direct peptide binding assays. GST-Grb2 or GST-Grap fusion protein (1.0 μM), radiolabeled tracer ([125I]ELFDDPSpYVNVQLDK, 10^5 cpm, ~35 fmol), and unlabeled peptides (BCR-pY177, KPFpYVN-VK; Shc-pY317, PSpYVNVQNL; and Shc-pY317 N319A, PSpYVAVQNL) were incubated together with glutathione beads as described previously (27, 72, 73).

The p36/38 bands could correspond to Lnk, a recently cloned, lymphoid tissue-restricted SH2 domain phosphoprotein with a predicted molecular mass of 34 kDa (37). In vitro, Lnk isolated from lymphocytes co-precipitates with Grb2, PLC-y1, and PI-3-kinase p85. It was reported that a single peptide (Cys-His-Leu-Arg-Ala-Ile-Asp-Gln-Tyr(P)-Thr-Pro-Leu-Ser-Gln-Leu) derived from Lnk blocks this interaction by binding SH2 domains of all three proteins. This would be unusual in that the peptide lacks appropriate SH2 domain binding motifs. We show that a related peptide (Asp-Asn-Gln-Tyr(P)-Thr-Pro-Leu-Ser-Gln-Leu) does not bind Grb2 or Grap directly (Fig. 6, A and B). Since the published protein sequence of Lnk has no consensus motif for Grb2 binding, the reported association between Lnk and Grb2 may be mediated indirectly through another protein.

Additional Grap Partners in T Cells: She, mSos, Sam68, and Dynamin—Since Grap and Grb2 SH2 domains have similar binding specificities, one would expect that Grap might co-precipitate with She, a major Grb2 SH2 domain partner in many cells. She is present in JMC-T cells, although the -fold increase in its phosphorylation is quite low (Fig. 5). In an additional experiment, Grap-Myc was immunoprecipitated from transfected JMC-T cells, and associated She was identified by immunoblotting (Fig. 7). T cell receptor activation does induce an association between She and Grap, although less than 2% of cellular She associates with Grap-Myc in these experiments. This is similar to the situation for Grb2 in Jurkat cells, where She association appears to be transient and of low stoichiometry (31, 35, 36).

Additional studies aimed at identifying potential SH3 domain partners of Grap. Two phosphoproteins interact with the SH3 domains of Grb2 in lymphocytes: the SH2 domain protein, SLP-76 (38, 39), and the protooncoprotein c-Cbl (19–21). SLP-76 and c-Cbl are usually detected in anti-phosphotyrosine immunoblots due to abundant phosphorylation. Although appropriate 76- and 120-kDa proteins were precipitated from activated Jurkat cell lysates by GST-Grb2, only p36/38 co-precipitated with GST-Grap (Fig. 4). These results suggest that SLP-76 and c-Cbl may not be major partners for Grap. Additional SH3 domain-associated proteins may not be detected as phosphoproteins. While mSos is the best characterized of the Grb2 SH3 domain-binding proteins, the p85 subunit of PI-3-kinase (40–42), dynamin (17, 18), Vav (16), c-Abl (43), and Sam68 (22, 44) also have proline-based motifs that may bind Grb2 SH3 domains. Grap-Myc was immunoprecipitated from transfected JMC-T cells, and the presence of certain candidates was probed by specific immunoblotting. mSos co-immunoprecipitates with Grap-Myc (Fig. 8). Furthermore, the amount of co-precipitated mSos increases with T cell receptor activation, analogous to the increase in Grb2/Sos association upon T cell activation (23, 24). Sam68 co-immunoprecipitates with Grap as well. However, Grap and Sam68 exhibit constitutive association, since there is little change in the amounts of co-precipitated proteins upon T cell activation. Dynamin also co-immunoprecipitates with Grap. As seen with Sos, the association between Grap and dynamin appears to be regulated by T cell activation. Previously, we showed that PI-3-kinase p85 co-precipitates with Grb2 and p36/38 in activated Jurkat cells (31). We have not detected p85 or c-Cbl in Grap-Myc immunoprecipitates (Fig. 8).

Sos, Sam68, and Dynamin Bind Grap via Its SH3 Domains—Since mSos is not tyrosine-phosphorylated and is known to bind the SH3 domains of Grb2, it is likely that it also binds the SH3 domains of Grap. Indeed, the C-terminal tail of Sos that contains four proline-rich motifs for SH3 domain recognition (BSos, Sos1 residues 1135–1322) binds Grap directly and with high affinity (Fig. 9B). Constitutive association between Grap-Myc and Sam68 suggests an SH3 domain-mediated interaction, as well (Fig. 9A). This association is decreased dramatically at low Sos tail concentrations (30 nM), indicating that Sam68 binds Grap through its SH3 domains. The association between Grap and dynamin is inhibited by the SH3 tail as well, suggesting that this interaction is also mediated by Grap SH3 domain binding to proline-rich motifs in dynamin. Like Sos, dynamin is known to bind the SH3 domains of Grb2 (17, 45). However, there is a suggestion in the literature that Sam68 might bind Grb2 via its SH2 domain (22) (this paper describes binding between Grb2 and GTPase-activating protein-associated p62, but, because the identity of these proteins has been confused, this is probably the protein referred to here.

**Fig. 7.** Low levels of She interact with Grap-Myc in activated Jurkat cells. JMC-T Jurkat cells were transiently transfected with a peptide-tagged Grap (Grap-Myc) or an empty vector (Mock) and stimulated (+) or not stimulated (−) with anti-CD3 antibody (αCD3). Proteins from 10^7 lysed cells were immunoprecipitated with an anti-Myc epitope antibody (αMyc) and detected by immunoblotting with anti-She antibodies (αShe). The amounts of She isoforms present in cell lysates are expressed as a percentage of the amount of lysate used for the immunoprecipitation experiment (i.e., less than 2% of the She present in activated JMC-T lysates co-precipitated with Grap-Myc.
as Sam68 (44)). To investigate further the mechanism of Grap and Grb2 binding with Sam68, mutated forms of the proteins were used in pull down experiments (Fig. 10). The Grb2 or Grap SH2 domain mutations do not prevent precipitation of Sam68 from Jurkat cell lysates, so neither protein binds Sam68 through its SH2 domain. In contrast, SH3 domain mutations resulted in much less precipitated Sam68. We conclude that Grap and Grb2 proteins bind Sam68 primarily via their SH3 domains and not their SH2 domains.

Additional Proteins in Grap Signaling Complexes: PLC-γ1 and Fyn—Grb2 and PLC-γ1 appear to interact with one another in activated Jurkat cells, either directly or indirectly (22, 31, 35). We have confirmed these findings using immobilized GST-Grb2 for protein precipitation and anti-PLC-γ1 antibodies for immunodetection (Fig. 11A). PLC-γ1 also precipitates with GST-Grap under these conditions, although less PLC-γ1 protein was precipitated by an equivalent amount of fusion protein. Additional studies with mutated proteins demonstrate that the Grap SH2 domain, and not its SH3 domains, is necessary for the effect. Weiss and colleagues (35) have suggested that Grb2 and PLC-γ1 both bind p36/38 rather than one another and that this is the basis for co-precipitation of Grb2 and PLC-γ1 (35). Our results with Grap and PLC-γ1 are consistent with a similar mechanism, particularly since an abundance of p36/38 precipitates with Grap, the Grap SH2 domain mediates both interactions, and PLC-γ1 has no Grb2/Grap SH2 domain binding site.

Sam68 interacts with and is phosphorylated by c-Src and Fyn (22, 46, 47). Having now identified Sam68 in T cells and shown that it associates with Grap and Grb2, it seemed possible that Fyn or perhaps Lck might be present in Grap signaling complexes. This was tested by immunoprecipitating Grap-Myc and immunoblotting with anti-Sam68 antibodies (aSam68), anti-dynamin (aDyn), anti-PI 3-kinase p85 (ap85), and anti-Cbl (aCbl) antibodies. The amount of lysate (right lanes) corresponds to 2% of that used in immunoprecipitation experiments.

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Grap in T Cells

FIG. 8. Grap-Myc binds mSos, Sam68, and dynamin but not PI 3-kinase p85 or c-Cbl. JMC-T Jurkat cells were transiently transfected with epitope-tagged Grap (Grap-Myc) or an empty vector (Mock) and stimulated (+) or not stimulated (−) with anti-CD3 antibody (αCD3). Proteins from 10^7 lysed cells were immunoprecipitated with an anti-Myc epitope antibody (aMyc) and detected by immunoblotting with anti-Sos antibodies (aSos), anti-Sam68 (aSam68), anti-dynamin (aDyn), anti-PI 3-kinase p85 (ap85), and anti-Cbl (aCbl) antibodies. The amount of lysate (right lanes) corresponds to 2% of that used in immunoprecipitation experiments.

FIG. 9. Grap associates with Sam68, dynamin, and Sos through its SH3 domains. JMC-T cells were transiently transfected with Grap-Myc or the empty vector (Mock) and stimulated (+) or not (−) with anti-CD3 antibody (αCD3). Lysates from 10^7 cells were incubated for 2 h at 4°C with recombinant, biotinylated mSos1 tail (BSos; residues 1135–1322) and anti-Myc antibody (aMyc) in a total volume of 0.5 ml. Proteins were precipitated with protein A-Sepharose, separated by SDS-PAGE, and identified by blotting with anti-Sam68 (aSam68), anti-dynamin (aDyn), or anti-Grap (aGrap) antibodies (A) or streptavidin-conjugated horseradish peroxidase (B). Control lanes contain varying amounts of lysate (A) or BSos (B).

Domains 97

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Fig. 10. Grb2 and Grap bind Sam68 through their SH3 domains. Jurkat cells were stimulated (+) or not (−) with anti-CD3 antibody (αCD3), lysed, and incubated (A) with immobilized GST-Grb2, or GST alone. Alternatively, lysates were incubated with wild-type (3-2-3) or mutated forms of GST-Grap (B) or GST-Grb2 (C). R86K mutations (3-2-3) block SH2 domain binding, while P49L and P206L mutations (Grb2) or P49L and P208L (Grap) mutations (3-2-3∗) block SH3 domain binding.

EF loop is WVV, while that of Grap is WEE, it is possible, for example, to unmask differences in binding specificity using peptides with acidic residues at positions C-terminal to asparagine.\(^3\) We do not know whether this finding has physiological relevance. Peptide binding specificities for Grap SH3 domains were not determined. However, residues lining the peptide binding pockets of Grb2 SH3 domains are known (52–54). Many corresponding residues of Grap are identical, suggesting similar binding specificities. Consistent with this suggestion, we have observed a similar subset of proteins binding the SH3 domains of Grap and Grb2.

Northern analyses show restricted expression of Grap in spleen, thymus, and circulating leukocytes, implying a function for Grap in lymphocytes. Its SH3-SH2-SH3 domain composition suggested that this function is in signal transduction. We show that the Grap protein is present in lymphocytes and acts downstream from antigen receptors (Fig. 12). In Jurkat cells, Grap associates avidly with p36/38 and to a lesser degree with Shc. These interactions require T cell receptor activation, phosphorylation of p36/38 or Shc, and a functional Grap SH2 domain. Grap also associates with mSos, the guanine nucleotide exchange factor for Ras, with Sam68, an RNA-binding protein potentially involved in cell division, and with dynamin, a guanosine triphosphatase involved in protein trafficking. Sam68 (22) in T cells, and Fyn binds Sam68 directly (22, 47).

Therefore, PLC-γ1 might be present in p36/38-Grap complexes with mSos, Sam68, and dynamin. Fyn may be found in p36/38-Grap-Sam68 complexes. Additional studies are needed to determine which of these ternary, quaternary, or higher order complexes actually form in activated lymphocytes.

Grap signaling complexes, we do not think that these are direct interactions. PLC-γ1 has been reported to bind p36/38 (35) and Sam68 (22) in T cells, and Fyn binds Sam68 directly (22, 47). Nevertheless, PLC-γ1 could be present in p36/38-Grap complexes with mSos, Sam68, and dynamin. Fyn may be found in p36/38-Grap-Sam68 complexes. Additional studies are needed to determine which of these ternary, quaternary, or higher order complexes actually form in activated lymphocytes.

Biological roles of Grap signaling complexes can be considered based on the biochemical functions of the SH3 domain-bound effector proteins and potential links to downstream cellular effects. IL-2 production provides a paradigm for studying immediate effects of T cell receptor activation on the nucleus (1, 3). Changes in IL-2 gene expression are influenced by intracellular calcium and the activation state of Ras. Significant headway has been made mapping molecular components of the Ca\(^{2+}\) and Ras pathways, which converge at the level of IL-2 gene transcription through synergistic effects on components of the nuclear factor of activated T cells (NF-AT) (55, 56). Activated Ras associates with Raf family serine/threonine kinases, leading sequentially to the phosphorylation and activation of MEK and the mitogen-activated protein kinases, ERK1 and ERK2.

The mitogen-activated protein kinases can translocate to the nucleus and regulate nuclear components of NF-AT (57, 58). Since Grap-Sos likely function much like Grb2-Sos as a guanyl nucleotide exchange factor for Ras, Grap may be an important link between the activated T cell receptor and the Ras/Raf/MEK/ERK cascade.

Calcium effects on NF-AT induction are likely through calcineurin, a two-subunit serine/threonine phosphatase whose activity is potently inhibited by cyclosporin-cyclophilin and FK506-FKB1 complexes (55, 56). The catalytic subunit binds calmodulin, and the regulatory subunit binds Ca\(^{2+}\). Cal-

\(^3\) G. Wolf and S. E. Shoelson, unpublished observations.
cineurin may regulate IL-2 gene expression by controlling nuclear translocation of a cytosolic component of NP-AT in response to T cell receptor-induced changes in Ca\(^{2+}\) flux. PLC-\(\gamma\)-1 has an important function in regulating local Ca\(^{2+}\) fluxes in T cells (6) through production of inositol 1,4,5-trisphosphate. Concomitant production of diacylglycerol potently activates PKC (59), although final effects of PKC pathways in T cells are unclear (2).

Grap-dynamin complexes may be involved in T cell receptor trafficking. Dynamin is a GTPase that contains a pleckstrin homology domain and a proline-rich region that binds SH3 domain proteins (17). These latter two regions may participate in the subcellular targeting of dynamin. As is true for many signaling proteins, much of what is known about dynamin function was originally inferred from studies in lower organisms. Dynamin is homologous with the Drosophila shibire gene product (60) and yeast Vps1p and Dnm1p proteins. Flies carrying the temperature-sensitive shibire allele exhibit a defect in protein endocytosis (61), and Vps1p and Dnm1p are involved in protein trafficking to the yeast vacuole (62, 63). In mammalian cells that overexpress GTP binding-defective mutants of dynamin, receptors sort to clathrin-coated pits, but the pits fail to constrict and invaginate (64, 65). In \textit{in vitro} dynamics assemble into stacked rings resembling the electron-dense collars that accumulate at nerve terminals of \textit{shibire} flies (66, 67). GTP hydrolysis correlates with a change in ring structure that may be associated with vesicle fusion. Grb2-dynamin complexes present in numerous cell types may play a role in receptor internalization (17, 18, 45). Since T cell activation leads to T cell receptor endocytosis and down-regulation (68, 69), Grap-dynamin complexes may have important functions in these events.

Grap and Grb2 also interact in T cells with Sam68, a protein with homology to the heterogeneous nuclear ribonucleoproteins (44, 70). Sam68 is phosphorylated by Src (or Src-like kinases) during G1 of the cellular interphase (22, 46, 47). It appears that the Src SH3 domain has a role in this, by binding one of the putative proline-rich binding motifs in Sam68. Selective phosphorylation by Src kinase during G1 implies a role in cell division, although cellular functions of Sam68 and biochemical effects of phosphorylation are unknown. Nevertheless, Sam68 binds RNA like a heterogeneous nuclear ribonucleoprotein and contains a KH domain common to this protein class. By extraposition, its function might be in the editing, stabilization, or metabolism of RNA. Since Sam68 may be a nuclear protein, Src and Sam68 may gain access to one another during breakdown of the nuclear envelope at the beginning of mitosis (71). In lymphocytes, Fyn and possibly Lck may take over functions of Src, to interact with and phosphorylate Sam68 during lymphocyte proliferation. Therefore, Grap-Sam68 and Grb2-Sam68 complexes may have roles in the entry of T lymphocytes into the cell cycle as a response to antigen receptor triggering.

Given its prominent expression in the thymus, Grap may function in lymphocyte differentiation and development. In this regard, Grap may have potential roles downstream from activated cytokine receptors that are distinct from its functions in T cell receptor signaling. Although lymphocytes contain numerous cytokine receptors, we have not tested this possibility. However, since Grap reportedly binds the stem cell factor receptor (c-Kit) isolated from MO7e cells and the erythropoietin receptor from the same cells that overexpress the receptor (8), Grap functions in lymphocytes is possible. These are obvious areas for future investigation.

It is important to consider why lymphocytes should have an auxiliary pathway, containing an extra substrate (p36/38) and linker protein (Grap), compared with most alternative cells, and why both pathways are activated by the T cell receptor. Many concepts in tyrosine kinase signal transduction, including the Grb2 paradigm, derive from studies with growth factor receptors. Growth factors and related hormones circulate freely or as dimers in solution and bind their receptor with high affinity and specificity. Typically one of these ligands (or ligand pairs) binds one receptor or receptor dimer. In this fashion, each ligand evokes a signaling response. Once many receptors have been occupied, a threshold is reached and the cell responds. Mechanisms for T cell activation are different. T cell receptors bind peptide/major histocompatibility complexes on the surface of antigen-presenting cells. These interactions are of low affinity and exhibit rapid dissociation, which enables each peptide/major histocompatibility complex to serially engage many T cell receptors (68). Since each ligand activates numerous receptors, a very few peptide/major histocompatibility complexes can trigger the lymphocyte response. Although certain functions of Grap and Grb2 must differ (e.g. Figs. 3 and 4), several effectors associate with both, including Sos, dynamin, Sam68, and (indirectly) PLC-\(\gamma\)-1. We speculate that the additive effects of Grap and Grb2 and the numerous associated effectors may help to amplify the unique T cell receptor signal inside the cell.

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\textbf{Figure 12. Schematic summary of potential Grap functions in T cell receptor signaling.}
