Vav1 and Ly-GDI Two Regulators of Rho GTPases, Function Cooperatively as Signal Transducers in T Cell Antigen Receptor-induced Pathways*

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The Rho family GTPases are pivotal for T cell signaling; however, the regulation of these proteins is not fully known. One well studied regulator of Rho GTPases is Vav1; a hematopoietic cell-specific guanine nucleotide exchange factor critical for signaling in T cells, including stimulation of the nuclear factor of activated T cells (NFAT). Surprisingly, Vav1 associates with Ly-GDI, a hematopoietic cell-specific guanine nucleotide dissociation inhibitor of Rac. Here, we studied the functional significance of the interaction between Vav1 and Ly-GDI in T cells. Upon organization of the immunological synapse, both Ly-GDI and Vav1 relocate to T cell extensions in contact with the antigen-presenting cell. Ly-GDI is phosphorylated on tyrosine residues following T cell receptor stimulation, and it associates with the Src homology 2 region of an adapter protein, Shc. In addition, the interaction between Ly-GDI and Vav1 requires tyrosine phosphorylation. Overexpression of Ly-GDI alone is inhibitory to NFAT stimulation and calcium mobilization. However, when co-expressed with Vav1, Ly-GDI enhances Vav1 induction of NFAT activation, phospholipase Cγ phosphorylation, and calcium mobilization. Moreover, Ly-GDI does not alter the regulation of these phenomena when coexpressed with oncogenic Vav1. Since oncogenic Vav1 does not bind Ly-GDI, this suggests that the functional cooperativity of Ly-GDI and Vav1 is dependent upon their association. Thus, our data suggest that the interaction of Vav1 and Ly-GDI creates a fine tuning mechanism for the regulation of intracellular signaling pathways leading to NFAT stimulation.

The Rho GTPase proteins participate in cellular processes such as cell cycle, movement and migration, metabolism, survival, proliferation, and differentiation (1–4). Rho GTPase proteins cycle between the GDP-bound inactive and GTP-bound active forms. Extracellular signals can affect Rho GTPase activity through at least three types of regulatory molecules: the GTPase-activating proteins that stimulate conversion from the GTP-bound form to the GDP-bound form; the GDP/GTP exchange factors (GEFs),1 which facilitate the shift from the GDP-bound form to the GTP-bound form; and the GDP dissociation inhibitors (GDIs), which block GDP dissociation from Rho GTPases, thus maintaining the inactive state (4). Despite accumulating experimental evidence, many details of the regulation of GDP/GTP exchange remain to be elucidated.

One of the well studied GEFs for Rho GTPases is Vav1, a hematopoietic cell-specific signal transducer protein (5, 6). Vav1 contains several characteristic structural motifs that enable its function as a signal transducer protein. In fact, Vav1 and the other ubiquitously expressed members of the Vav family of proteins (Vav2 and Vav3) are the only known Rho GEFs that have SH2 domains, suggesting that their GEF activity is regulated by tyrosine phosphorylation (7–10). One of the best studied roles of Vav1 is as a signal transducer in activated T cells. TCR stimulation with antigen or with cross-linking antibodies initiates a complex signaling cascade. The earliest event in this cascade is the activation of multiple cytoplasmic tyrosine kinases, including the Src family tyrosine kinases Lck and Fyn. This leads to the phosphorylation of the immunoreceptor tyrosine activation motifs on the TCR (11). The phosphorylated tyrosines in these immunoreceptor tyrosine activation motifs associate with the SH2 domains of ZAP-70, another cytoplasmic tyrosine kinase (12). Src tyrosine kinases also phosphorylate and activate ZAP-70, leading to the tyrosine phosphorylation of downstream signaling proteins, including Vav1 (5, 6). TCR-induced tyrosine phosphorylation of Vav1 leads to activation of its GEF function toward Rac, its preferred target GTPase (9, 10). This signaling cascade results in the activation of the nuclear factor of activated T cells (NFAT), which plays a critical role in the regulation of many genes including interleukin-2 (13). The cardinal role of Vav1 in NFAT induction is inferred from numerous in vitro and in vivo experiments. Overexpression of Vav1 was shown to induce NFAT stimulation in T cells (13). This effect was enhanced upon stimulation of TCR (13). Furthermore, Vav1-deficient cells fail to mobilize calcium and reorganize the cytoskeleton, events that are related to Vav1 GEF activity and are important for NFAT stimulation (14–18). The precise mechanism by which Vav1 induces NFAT is not yet entirely resolved. It is clear that Vav1’s function as a GEF toward Rac is important for this process. However, it has been suggested that Rac-independent Vav1 activities are also involved (5). In addition to its

1 The abbreviations used are: GEF, GDP/GTP exchange factors; GDI, GDP dissociation inhibitor; SH2 and SH3, Src homology 2 and 3, respectively; TCR, T cell receptor; NFAT, nuclear factor of activated T cells; Ab, antibody; mAb, monoclonal antibody; PLC, phospholipase C; APC, antigen-presenting cell; WT, wild type; DIC, differential interference contrast.
role in T cell activation, Vav1 is also involved in numerous other immune functions such as T-cell development, differentiation, and cell cycle control (19).

Vav1 was first isolated as an oncogene (20). However, wild-type Vav1 transforms NIH3T3 fibroblasts only when it is overexpressed. It is significant to note that converting wild-type Vav1 to an oncogene involves mutations/deletions in its amino terminus (21, 22). Thus, removal of 66 residues from the amino terminus, mimicking the mode of activation of the originally isolated oncogene, is sufficient to induce transformation by Vav1 in murine fibroblasts. A more potent transforming form of Vav1 is obtained when an even larger (186 residues) region is removed (5). The amino terminus of Vav1 is not only necessary for regulating its transforming activity but was also found to be important for Vav1-mediated NFAT transcription. Overexpression of wild-type Vav1 in Jurkat T cells leads to activation of NFAT; however, the truncated oncogenic proteins, Vav1-66 and Vav1-186, do not cause any changes in NFAT transcription, even when the TCR is stimulated (5, 13). The amino terminus region resembles a calponin homology region yet is unlikely to directly associate with F-actin, since two such regions in tandem are needed for association with actin (23). Since this region is devoid of any catalytic activity and is rich in α-helical content, it seemed plausible that it might participate in protein-protein interactions. Indeed, we have recently demonstrated that the amino terminus region of Vav1 interacts in vitro and in vivo with another potential regulator of Rho GTPases, the hematopoietic-specific GDI protein, Ly-GDI (24).

GDIs regulate Rho GTPase activity by inhibiting GDP dissociation, promoting the inactive form (4, 25). The ubiquitously expressed Rho-GDI also appears to function as a chaperone for the Rho GTPases, shuttling them between the cytosol and the membrane. In addition, Rho-GDI blocks both intrinsic and GAP-stimulated GDP hydrolysis (4, 25). Whether all of these functions are also carried out by Ly-GDI in T cells is still an open question. Stimulation of T lymphocytes and myelomonocytic cells with phorbol esters leads to phosphorylation of Ly-GDI on serine/threonine residues, raising the possibility that Ly-GDI is involved in signaling pathways in these cells (26, 27). In addition, Ly-GDI is constitutively phosphorylated on tyrosine residues in neutrophils (28). It is not known if the function of Ly-GDI in these cells is also regulated by extracellular signals.

The association between Vav1 and Ly-GDI is particularly intriguing because, theoretically, these two proteins should have opposite effects on the activity of small Rho GTPases. Since the function of Ly-GDI in T cells is largely unknown, we examined its activity, localization, response to TCR stimulation, and potential effect on the function of Vav1 in T cells. Here, we show for the first time that the distribution of Ly-GDI is altered in stimulated T cells and accumulates with Vav1 in the membrane extensions in the periphery of the "immunological synapse." Ly-GDI exhibits several characteristics of a protein involved in signaling. First, Ly-GDI is phosphorylated on tyrosine residues following TCR stimulation. Second, it associates with the SH2 region of an adapter protein, Shc. Third, Ly-GDI interacts with Vav1 when signaling is enabled in T cells. Ly-GDI can block calcium mobilization in Jurkat cells; however, surprisingly, rather than counteracting the effects of Vav1, Ly-GDI further enhances the induction of NFAT when the TCR is stimulated in T cells overexpressing Vav1. Thus, in T cells, these two regulators of Rho GTPases appear to function cooperatively as signal transducers in the TCR pathway and may be involved in the cytoskeletal reorganization required for formation of the "immunological synapse."
and Fura Red-AM (10 μg/ml; Molecular Probes) for 45 min at 37 °C. Cells were then rinsed and incubated for 20 min at room temperature. Each sample was left untreated for 30 s, and then cells were stimulated by the addition of anti-human CD3 and anti-mouse IgG. Calcium mobilization was determined by the intensity ratio of Fluo-3/Fura Red fluorescence over time as recorded by flow cytometry.

RESULTS

Following T Cell Activation, Vav1 and Ly-GDI Accumulate in F-actin-rich Membrane Extensions That Protrude in the Contact Zone with the APC—Upon stimulation of the T cell, redistribution of signaling molecules occurs leading to the formation of an organized “immunological synapse” at the contact between a T cell and an APC (32, 33). High resolution immunofluorescence imaging of T cell-APC conjugates allows the visualization of two distinct regions within the immunological synapse: the central zone and the peripheral zone. The central zone (c-SMAC) contains the TCR, surface co-stimulatory receptors such as CD28, and intracellular signaling molecules such as protein kinase CD, whereas the peripheral zone (p-SMAC), is enriched for the integrin LFA-1 and the actin-binding protein talin (34–36). It is widely believed that reorganization of the immunological synapse is required for TCR signaling (32, 33).

F-actin plays a critical role in this reorganization (32). The polymerization of F-actin is regulated by Rac (37); therefore, it was of interest to establish the involvement of the Rac regulators, Vav1 and the GDIs, in the organization of the immunological synapse.

We used confocal microscopy to determine the localization of Vav1 and Ly-GDI during the formation of the immunological synapse. This approach allowed us to determine the specific subcellular localization of these proteins in Jurkat T cells, information that could not be achieved by biochemical approaches. Jurkat cells were incubated with APCs either unpulsed (control) or prepulsed (activated) with S. enterotoxin E superantigen and the intracellular localization of Vav1 and Ly-GDI was determined with two-color immunofluorescence. As shown in Fig. 1, an accumulation of Vav1 in membrane extensions in the contact area with the APC was observed at 5 min (B), was maximal at 15 min (C), and was mostly undetectable at 30 min (D). Likewise, Ly-GDI accumulated in these membrane extensions overlapping Vav1 accumulation in these areas and following the same kinetics (Fig. 1, B–D). In addition to the accumulation in membrane extensions, a minute fraction of Vav1 was also observed in the central zone of the junction, whereas Ly-GDI was mainly seen in the extensions and not in the center. In contrast, in conjugates formed by T cells and unpulsed APCs, no relocalization of Vav1 or Ly-GDI was observed at any of the incubation times (Fig. 1A and data not shown). Contrary to Ly-GDI, no accumulation of Rho-GDI in the contact area seemed to occur (Fig. 2). Thus, the same fluorescence density of Rho-GDI was observed in the membrane extensions in contact with the APC and in other areas of the cell (Fig. 2, B–D). These data indicate that Vav1 and Ly-GDI, but not Rho-GDI, translocate to membrane extensions in contact with the APC in response to superantigen stimulation.

Their accumulation was transient and correlated with strong T contact with the APC in response to superantigen stimulation. GDI, but not Rho-GDI, translocate to membrane extensions in presence of Vav1, Ly-GDI, and F-actin. As shown in Fig. 3B, the membrane extensions in contact with the APC that displayed accumulation of Vav1 and Ly-GDI were also enriched in F-actin. Therefore, a correlation seems to exist between accumulation of Vav1 and Ly-GDI and increased actin polymerization in these subcellular areas. In contrast to activated cells, nonactivated T cell-APC conjugates did not display accumulation of F-actin, Vav1, and Ly-GDI in the contact area (Fig. 3A).

Comparison of staining for TCR-CD3, Vav1, and Ly-GDI revealed that Vav1 and Ly-GDI accumulations occurred at the periphery of the T cell-APC contact site, whereas the TCR-CD3 clustered in the center. Some overlap between TCR-CD3 and Vav1 staining was observed in the center of the contact zone, although Vav1 was mostly present in the peripheral cell extensions (Fig. 3D).

Altogether, these immunolocalization experiments indicate that Vav1 and Ly-GDI accumulate in the contact zone between T cells and stimulatory APCs within membrane extensions that transiently form and enlarge the contact area between the cells. These extensions are also enriched in F-actin, suggesting that a correlation exists between Vav1 and Ly-GDI accumulation and increased actin dynamics in these cellular areas.

Vav1 and Ly-GDI Associate in the Cytoplasm—Activation of the exchange of GDP for GTP on Rho family GTPases is accompanied by their intracellular translocation from the cytoplasm to the plasma membrane. The subcellular localization of the regulators of Rho GTPases is less well established, although Vav1 was shown to be recruited to the plasma membrane in response to activation of Fe receptors (38). The GDI regulators,
Ly-GDI and Rho-GDI, are considered to be predominantly cytosolic (25). Our confocal microscopy results raised the possibility that Vav1 and Ly-GDI proteins are present in the same cellular microenvironment upon activation of Jurkat T cells (Figs. 1–9), suggesting that these proteins might cooperate. To explore this possibility, we first established whether Vav1 and Ly-GDI interact in specific subcellular compartments in Jurkat T cells.

Vav1, Ly-GDI, and Rho-GDI were found predominantly in the cytoplasm (Fig. 4A). Immunoblotting with anti-CD28 mAbs and anti-actin mAbs verified the purity of the particulate (membrane) and cytoplasmic fractions and ruled out significant cross-contamination (Fig. 4A). We demonstrated previously that Vav1 and Ly-GDI associate in T cells both in vitro and in vivo (24). To further investigate the subcellular localization of their interaction, cytoplasmic and particulate (membrane) fractions of Jurkat T cells were immunoprecipitated with anti-Vav1 Abs (Fig. 4B) and immunoblotted with either anti-Vav1 mAbs (Fig. 4B, upper panel) or with anti-Ly-GDI Abs (Fig. 4B, lower panel). Co-immunoprecipitation of Vav1 and Ly-GDI was observed in the cytoplasmic fraction and was not apparent in the particulate fraction (Fig. 4B). In contrast, Rho-GDI, previously shown to bind to Vav1 in vitro (24), does not associate with Vav1 in Jurkat T cells in vivo (Fig. 4C, lower panel, lane 6 versus lane 4). Longer exposures of the autoradiograms did not reveal the presence of Rho-GDI in the immunoprecipitates of Vav1. Thus, in vivo, Vav1 discriminates between the GDI proteins and binds only to the hematopoietic-specific GDI, Ly-GDI. These results imply that Vav1 and Ly-GDI might cooperate in T cells.

To determine whether the association between Vav1 and Ly-GDI is regulated by T cell activation, Jurkat T cells transiently transfected with Vav1 and Ly-GDI were stimulated with anti-CD3 mAbs (Fig. 4D). Cell lysates were immunoprecipitated with anti-Vav1 Abs and immunoblotted with anti-Ly-GDI Abs. Co-immunoprecipitation of Vav1 and Ly-GDI was observed in control Jurkat T cells and increased significantly following TCR stimulation with anti-CD3 mAbs (Fig. 4D, right panel, lane 2 versus lane 1). This constitutive interaction might be the result of already tyrosine-phosphorylated proteins such as Vav1 in a cell line such as Jurkat.

TCR stimulation induces tyrosine phosphorylation of Vav1 (39), and Vav1 activity is regulated by tyrosine phosphorylation (5). To explore the role of tyrosine phosphorylation in the Ly-GDI/Vav1 association, cells were treated with the protein-tyrosine kinase inhibitor herbimycin A prior to the addition of anti-CD3 mAbs (Fig. 4D). No significant co-immunoprecipitation of Ly-GDI and Vav1 was observed in herbimycin A-treated Jurkat cells even following stimulation with anti-CD3 (Fig. 4D, right panel, lanes 3 and 4). Our results demonstrate that tyrosine phosphorylation plays a role in the interaction between Vav1 and Ly-GDI.

Ly-GDI as a Potential Signal Transducer Protein—Both the fact that tyrosine phosphorylation is important for the association between Vav1 and Ly-GDI (Fig. 4) and the fact that Ly-GDI contains consensus sequences for tyrosine phosphorylation prompted us to investigate whether Ly-GDI is tyrosine-phosphorylated following engagement of the TCR. To accomplish this, Jurkat T cells were stimulated with anti-CD3 mAbs, a treatment known to induce tyrosine phosphorylation of nu-
merous signaling molecules, including Vav1 (39). Total lysates and anti-phosphotyrosine immunoprecipitates from these cells were immunoblotted with anti-Ly-GDI Abs or anti-Rho-GDI Abs. The results suggest that Ly-GDI is tyrosine-phosphorylated in response to anti-CD3 mAbs, whereas Rho-GDI is not (Fig. 5A, lanes 1, 2, 5, and 6). Since anti-Tyr(P) mAbs may immunoprecipitate Ly-GDI indirectly, we used a more direct approach that also eliminated the cross-reactivity of light chains of Abs (Fig. 5B). Jurkat T cells were transiently transfected with a Myc-tagged pSec/Ly-GDI plasmid and then were either left nonstimulated (lanes 1 and 2) or stimulated with anti-CD3 mAbs (lane 3). Cells were then lysed and immunoprecipitated with anti-Myc mAbs (lanes 2 and 3) or nonimmune sera (lane 1), separated on SDS-PAGE, and immunoblotted with either anti-Ly-GDI Abs or anti-Tyr(P) mAbs (Fig. 5B). The results of this experiment verify that Ly-GDI exhibits an increase in tyrosine phosphorylation in activated Jurkat cells (lane 3 versus lane 2). Using a computer program to screen the deduced amino acids of the encoded Ly-GDI and Rho-GDI proteins for consensus tyrosine phosphorylation sites, we located four tyrosines that have a high probability of being phosphorylated in Ly-GDI (Tyr172, Tyr153, Tyr125, and Tyr156). Nevertheless, a fundamental difference exists between these two GDI proteins. Whereas Ly-GDI is tyrosine-phosphorylated in activated T cells, Rho-GDI is not.

Since Ly-GDI is phosphorylated on tyrosine residues in T cells, it is conceivable that it will associate with SH2-containing proteins, a characteristic feature of tyrosine-phosphorylated proteins. The motif-based profile scanning approach developed by Yaffe et al. (40) predicted that Ly-GDI would associate with the SH2 region of Crk and Shc. The SH2 region of Shc was produced as glutathione S-transferase fusion protein in a bacterial expression system, purified, bound to glutathione-Sepharose beads, and used in a binding assay with Ly-GDI. Ly-GDI associated with the SH2 region of Shc (Fig. 5C, lanes 2–4). Stimulation of TCRs with anti-CD3 mAbs and with both anti-CD3 and anti-CD28 Abs increased binding of Ly-GDI to Shc (Fig. 5C, lanes 3 and 4 versus lane 2). Ly-GDI also co-immunoprecipitates with Shc in a TCR-induced fashion (Fig. 5D, right panel, lane 2 versus lane 1). Based on the fact that Ly-GDI is tyrosine-phosphorylated upon TCR stimulation and it associates with the SH2 region of the adapter protein Shc in a TCR-induced manner, we conclude that Ly-GDI is a protein that participates in signaling cascades in T cells.

**Influence of GDI Proteins on NFAT Stimulation by Vav1**—Wild-type Vav1 appears to be necessary for full activation of the transcription factor NFAT following TCR stimulation (13). It has been suggested that the ability of Vav1 to stimulate NFAT activation depends on its ability to function as a GEF toward Rac as well as on converging signals from the Ras pathway (5). We wished to analyze whether Ly-GDI influences the NFAT induction by Vav1 overexpression in T cells. The rationale for this experiment was based on the following. First, Ly-GDI is considered to be a GDI for the Rho GTPase proteins, including Rac; therefore, it might influence Rac-associated downstream functions such as NFAT activity (4, 5). Second, Ly-GDI associates with wild-type Vav1 but not with the Vav1 amino terminus oncogenic deletion mutants (24). The amino terminus of Vav1 seems to control NFAT stimulation by an as yet unknown mechanism. Third, Ly-GDI was found by us to become phosphorylated on tyrosine residues following TCR stimulation (Fig. 5), suggesting that it might play a role in T cell signaling pathways.

To investigate the role of Ly-GDI in Vav1-induced NFAT activation, Jurkat T cells were cotransfected with a luciferase reporter driven by a promoter containing NFAT binding sites and several plasmids encoding Vav1, Vav1 mutants, Ly-GDI, or Rho-GDI (outlined in Fig. 6). Luciferase activity in cell extracts was assessed following activation of the TCR by anti-CD3 mAbs. As expected, in cells transfected with WT Vav1, luciferase activity is significantly increased upon TCR activation (Fig. 6, lane 4 versus lane 3). Surprisingly, the Vav1-induced increase in luciferase activity was further enhanced when Ly-GDI was co-introduced into Jurkat T cells (lane 6 versus lane 4) despite the fact that Ly-GDI inhibits the luciferase activity by 70% when transfected in the absence of Vav1 (lane 8 versus lane 4). No luciferase induction was observed when Ly-GDI was co-transfected with the Vav1 amino terminus oncogenic deletion mutant (OncVav1; lanes 9 and 10).
phospho-PLC Unlike WT Vav1, oncogenic Vav1 does not lead to an increase in (anti-Shc Abs and immunoblotted as indicated (proteins were separated on SDS-PAGE and then immunoblotted with anti-Ly-GDI Abs. D2 immunoprecipitated with either preimmune serum (p.i.; lane 1) or with anti-Myc Abs (lanes 2 and 3). The blots were hybridized with either anti-Ly-GDI or anti-Tyr(P) as indicated. C, bacterially expressed glutathione S-transferase (lane 1) and glutathione S-transferase-SH2She (lanes 2–4) were bound to glutathione beads. The bound proteins were then incubated with lysates of Jurkat T cells that were either activated with anti-CD3 mAbs for 2 min (lanes 1 and 3) or anti-CD3 and anti-CD28 mAbs incubated for 15 min (lane 4) or left untreated (lanes 2). The bound proteins were separated on SDS-PAGE and then immunoblotted with anti-Ly-GDI Abs. D, Jurkat T cells were either nontreated (lane 1) or stimulated with anti-CD3 Abs (lane 2). Lysates of these cells were either immunoblotted with anti-Tyr(P) or immunoprecipitated with anti-Shc Abs and immunoblotted as indicated (right panel). The level of Ly-GDI in the cell lysates was determined by immunoblotting as indicated (right panel). The figure depicts one representative experiment of four performed.

Contrary to the effect of Ly-GDI, Rho-GDI blocks the Vav1-induced increase in luciferase activity (lane 14 versus lane 4).

The fact that oncVav1 neither associates with Ly-GDI nor enhances NFAT whereas Vav1 does both suggests that Ly-GDI association is required for Vav1’s ability to stimulate NFAT. The synergy of Vav1 and Ly-GDI in the co-transfection experiments supports this notion.

Ly-GDI, Vav1, and Induction of PLCγ—Vav1 was recently shown to regulate the activation of PLCγ by phosphorylation and GEF-dependent pathways, events that are critical for proper calcium responses and various signal transduction pathways in hematopoietic cells (41, 42). We next tested the possibility that Ly-GDI contributes to this pathway and therefore influences Vav1-induced NFAT stimulation. As shown in Fig. 7A, Jurkat T cells transfected with Vav1 displayed increased tyrosine phosphorylation of PLCγ compared with cells transfected with empty vector (Fig. 7A, lane 4 versus lane 2). This increase was augmented when Ly-GDI was co-transfected with Vav1 (Fig. 7A, lane 6 versus lane 4). Interestingly, unlike WT Vav1, transfection with oncogenic Vav1 does not lead to an increase in phospho-PLCγ (lane 8 versus lane 4). When Ly-GDI is co-transfected with oncogenic Vav1, levels of phospho-PLCγ are not significantly different from levels in cells transfected with oncogenic Vav1 alone (lane 10 versus lane 6). In fact, the level of phospho-PLCγ remained similar to that observed in cells transfected with vector only (lanes 1 and 2). Interestingly, unlike WT Vav1, oncogenic Vav1 does not lead to an increase in phospho-PLCγ (lane 8 versus lane 4).

Several important conclusions can be drawn from these experiments. First, WT Vav1 differs from oncogenic Vav1 in its ability to lead to activation of PLCγ. Second, Ly-GDI can participate in PLCγ activation only when it is expressed with WT Vav1, with which it associates, but not when it is expressed with oncogenic Vav1, with which it does not interact.

Ly-GDI and Calcium Mobilization—NFAT activation is dependent on calcium mobilization following TCR stimulation (43). Several proteins including Vav1 and Cdc42/Rac1 were shown to play a critical role in receptor-stimulated Ca2+ mobilization; therefore, it seemed reasonable that Ly-GDI, a regulator of Rho GTPases, might also be involved and thus affect NFAT stimulation. Changes in calcium mobilization of TCR-stimulated cells transfected with vector alone (red line), Vav1 (blue line), Vav1 and Ly-GDI (green line), and Ly-GDI (black line) was determined by the ratio of fluorescence intensities of Fura-3 and Fura Red over time. As shown in Fig. 7B, Jurkat T cells transfected with Ly-GDI exhibited significant reduced calcium mobilization compared with cells transfected with the empty vector. In contrast, transfection with Vav1 led to an increase in calcium mobilization that was larger, more rapid, and more sustained than that observed in cells transfected with vector only. Cells transfected with both Vav1 and Ly-GDI exhibit a pattern similar to that obtained in cells transfected with Vav1 alone, although some increase in calcium mobilization can be noted. Thus, transfection of Jurkat cells with Vav1 overrides the inhibition in calcium mobilization exerted by Ly-GDI. Unlike WT Vav1, oncogenic Vav1 appears to exert an inhibitory effect on calcium mobilization; Jurkat T cells transfected with oncogenic Vav1 display reduced calcium mobilization similar to that observed in cells transfected with Ly-GDI (Fig. 7C). There is no combinatorial effect of transfecting Jurkat cells with both Ly-GDI and oncogenic Vav (Fig. 7C).

Our results demonstrate that Ly-GDI and WT Vav1 have
opposite effects on calcium mobilization; Ly-GDI is inhibitory, and Vav1 is stimulatory. Furthermore, WT Vav1 not only reverses the Ly-GDI-generated inhibition, but its positive effect is not hindered by the presence of Ly-GDI.

**DISCUSSION**

The regulation of Rho GTPase activity is integral to many basic cellular functions and can be controlled by extracellular signals (1–4). Regulatory proteins for small Rho GTPases include GEFs and GDIs, which have opposite effects on GTPase activity. Vav1 exhibits a regulated GEF activity in T cells following engagement of the TCR (5). Intriguingly, we found recently that a potential negative regulator of Rho GTPases, Ly-GDI, associates with Vav1 in vivo and in vitro (24). The function and regulation of the hematopoietic-specific Ly-GDI in stimulated T cells have not been well described.

Here, we show that the distribution of both Vav1 and Ly-GDI is altered in stimulated T cells, with both proteins accumulating in the membrane extensions of the "immunological synapse" (32–36). The formation of the immunological synapse at the contact between a T cell and an APC was recently suggested to play a cardinal role in signaling in activated T cells. Thus, determining the subcellular location of all known signaling proteins during engagement of the TCR appears to be critical to understanding the TCR-induced signaling cascade. The relocalization of Ly-GDI and Vav1 following immunological synapse formation was dependent upon activation of the APCs. The distribution of these two Rho GTPase regulators displayed a partial overlap with one distinct difference. Vav1 accumulated mainly in the extensions of the T cells at the contact area with the APC; however, a minute amount was also

**FIG. 6.** Effect of Ly-GDI and Rho-GDI on the stimulation of NFAT by wild-type Vav1 and oncogenic Vav. Jurkat tag cells were electroporated with various plasmids: pSec vector (lanes 1 and 2); pEFVav1 (pEF115; lanes 3 and 4), pEFVav1 and Ly-GDI (lanes 5 and 6), Ly-GDI (lanes 7 and 8), oncogenic Vav (OneVav; pEF67) and Ly-GDI (lanes 9 and 10), Vav1 and Ly-GDI (lanes 11 and 12), and Rho-GDI (lanes 13 and 14). All of these plasmids were electroporated together with β-galactosidase and NFAT-luciferase reporter gene plasmids as described under "Experimental Procedures." Eighteen hours following electroporation, cells were activated with anti-CD3 and lysed 8 h later. Luciferase activities were normalized to β-galactosidase activities to correct for transfection efficiency as described under "Experimental Procedures." -Fold induction refers to the division of values obtained in each lane by the ones obtained with vector alone in nonactivated conditions. Histograms represent the mean ± S.E. of duplicate values obtained in eight experiments performed. The paired t test was used to evaluate the statistical significance in luciferase activity between the following: (a) cells transfected with vector alone (lane 2) versus cells transfected with WT Vav1 (lane 4; p < 0.002); (b) cells transfected with WT Vav1 (lane 4) versus cells transfected with the combination of WT Vav1 and Ly-GDI (lane 6; p < 0.025); (c) cells transfected with Ly-GDI (lane 8), oncVav1 and Ly-GDI (lane 10), WT Vav1 and Rho-GDI (lane 12), and Rho-GDI versus cells transfected with WT Vav1 (lane 4; p < 0.002). The inset depicts the levels of exogenous transfected proteins, as revealed by Western blots. The levels of Ly-GDI and Rho-GDI from lysates of transfected cells (lanes 6 and 12, respectively) were assessed by immunoblotting with anti-Ly-GDI Abs and anti-Rho-GDI Abs, respectively. The identity of the transfected proteins was verified by immunoblotting with anti-Myc mAbs (data not shown). The levels of Vav1 and the oncogenic Vav1 were determined by anti-Vav mAbs. The exposure of the autoradiogram illustrates the level of the transfected proteins (verified by anti-Myc mAbs).

**FIG. 7.** Phosphorylation of PLCγ (A) and calcium mobilization (B and C) are influenced by Vav1 and Ly-GDI. A, Jurkat cells were transfected with empty vector (lanes 1 and 2), WT Vav1 (lanes 3 and 4), Vav1 and Ly-GDI (lanes 6 and 7), oncogenic Vav (Vavonc; lanes 7 and 8), and Vavonc and Ly-GDI (lanes 9 and 10) and were either nontreated (lanes 1, 3, 5, 7, and 9) or stimulated with anti-CD3 (lanes 2, 4, 6, 8, and 10). Cell lysates were then immunoprecipitated (IP) with anti-PLCγ and immunoblotted (WB) with anti-phospho-PLCγ (Tyr783) Abs. The levels of PLCγ in each sample were determined by immunoblotting with anti-PLCγ Abs. The formation of the immunological synapse at the contact between a T cell and an APC was recently suggested to play a cardinal role in signaling in activated T cells. **B,** Jurkat T cells were transfected with vector, Vav1, Vav1 and Ly-GDI, and Ly-GDI as indicated. Cells were loaded with Fluo-3/AM and Fura Red as indicated under "Experimental Procedures." Cells were stimulated by anti-CD3 Abs and anti-mouse IgG. Data shown are the ratios of Fluo-3 and Fura Red fluorescence measured over time by a flow cytometry. One representative experiment of three performed. C, a similar experiment to the one described above was performed, but instead of WT Vav1, oncogenic Vav1 was used as indicated. The figure depicts one representative experiment of three performed.

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present in the central contact zone. Ly-GDI also concentrated at the T cell extensions but was excluded from the central zone. We did not observe any preferential accumulation of Rho-GDI in the extensions or the central zone. This was consistent with our biochemical results showing no association of Rho-GDI with Vav1 with or without TCR stimulation, suggesting that the Vav1-Ly-GDI association is very specific. It is noteworthy that in T cells exhibiting synapses that look more mature, with strong and compact TCR accumulations, smaller protrusions can be found (data not shown). In these cases, the accumulation of Vav1 and Ly-GDI was weaker, suggesting that Vav1 and Ly-GDI relocation precede TCR clustering. Furthermore, we showed that the relocation of Vav1 and Ly-GDI coincided with a strong accumulation of F-actin in the same subcellular areas. This suggests a link between the accumulation of Vav1 and Ly-GDI and increased actin dynamics in those cellular regions. Experiments with T cells from mice deficient for Vav1 also indicated that Vav1, Rac, and actin cytoskeleton participate in the lipid raft formation that is needed for the immunological synapse (44). Furthermore, using a green fluorescent protein-tagged binding domain of WASP, that binds the active GTP-bound Cdc42, a Rho family GTPase, it was demonstrated that Cdc42 localizes along the T cell/APC contact site in an antigen-dependent manner (45), thus exhibiting the role of GTPases in the organization of the immunological synapse. Taken together, our results support the common notion that Vav1 and possibly Ly-GDI are involved in cytoskeletal rearrangement that occurs during the formation of the immunological synapse.

The relocation of Ly-GDI during the organization of the immunological synapse suggested to us that it might be regulated by engagement of the TCR. Our results illustrate for the first time that induction of the TCR leads to increased tyrosine phosphorylation of Ly-GDI but not of Rho-GDI (Fig. 5). In addition, TCR stimulation leads to association of Ly-GDI, but not Rho-GDI, with the SH2 domain of the adapter protein Shc (Fig. 5). Moreover, the association of Ly-GDI with Vav1 depends on tyrosine phosphorylation following TCR activation (Fig. 4). Consequently, our results suggest that Ly-GDI is specifically activated by TCR engagement and participates in transmitting extracellular signals in T cells.

Stimulation of T cells results in activation of NFAT, which is involved in the production of interleukin-2 and in relaying signals that coordinate immune responses (46–48). Stimulation of TCR leads to calcium mobilization and activation of the phosphatase calcineurin (49, 50). Calcineurin dephosphorylates NFAT, enabling it to translocate to the nucleus, where it binds specific DNA sequences and enhances transcription by binding to the amino terminus of Vav1 promote NFAT phosphorylation or truncation of a Tyr174 Lck kinase recognition site within the N terminus could therefore result in stimulation of GEF activity (56). However, this result does not fully explain the complex and diverse results obtained with amino terminus Vav1 mutants. Specifically, if this hypothesis was correct, the Vav1 Y174F mutant should exhibit a phosphorylation-independent GEF activity. However, Vav1 Y174F retains phosphorylation-dependent exchange activity which can stimulate NFAT activity once the TCR is activated (57, 58). Thus, this explanation of the role of the amino terminus of Vav1 in NFAT induction is unsatisfactory and might imply that other explanations for the role of the amino terminus of Vav1 for NFAT induction must exist.

Ly-GDI associates with the amino terminus region of Vav1 (24). Could Ly-GDI be part of the missing link between Vav1 and NFAT activation? Ly-GDI does increase the activation of NFAT in T cells when co-expressed with Vav1, yet it does not activate NFAT when co-expressed with the oncogenic Vav1, which does not bind Ly-GDI (Fig. 6) (24). Thus, the ability of Vav1 to induce NFAT seems to be correlated with its ability to associate with Ly-GDI. When co-expressed with Vav1, Ly-GDI also enhances Vav1’s effects on PLCγ phosphorylation and possibly calcium mobilization; however, it does not alter the regulation of these phenomena by oncogenic Vav1. Again, this strongly supports the hypothesis that Vav1 and Ly-GDI must physically associate to exert their cooperative functions.

How could the association of Ly-GDI with the amino terminus of Vav1 promote NFAT activation? Ly-GDI seems to inhibit NFAT stimulation (Fig. 6), which correlates with its capacity to block calcium mobilization (Fig. 7). This activity most probably stems from its ability to inhibit members of the Rho GTPases. Indeed, it was recently shown that a strong structural similarity exists between Ly-GDI and Rho-GDI (59–61). Furthermore, several in vitro studies suggest that Ly-GDI is as efficient as Rho-GDI in inhibiting GDP to GTP exchange on Rho GTPase proteins (62). Yet, conflicting studies claim that Ly-GDI is less efficient than Rho-GDI in its activity as a GTPase inhibitor (26, 63). Nonetheless, apparently the inhibitory activity of Ly-GDI toward Rho GTPases is sufficient to block calcium mobilization as well as NFAT stimulation. Moreover, it is possible that Ly-GDI inhibits an unknown hematopoietic cell-specific GTPase more potently than it inhibits known Rho GTPases.

Cdc42 and Rac were shown to act upstream of the calcium influx pathway. Cells expressing the dominant active mutants of Cdc42 and Rac exhibit elevated levels of antigen-stimulated inositol 1,4,5-trisphosphate production, leading to calcium mobilization upon activation (64, 65). When Vav1 and Ly-GDI are concomitantly overexpressed in Jurkat T cells, both calcium mobilization and NFAT are stimulated, thus indicating that Vav1 overrides the inhibition exerted by Ly-GDI. It is conceivable that the interaction between these proteins extracts Ly-GDI away from its target GTPase and enables Vav1 to function more efficiently as a GEF. Indeed Rho-GDI, which does not associate with Vav1, reduced NFAT activation regardless of the expression of Vav1 (Fig. 6). This result indicates that the ability of Rho-GDI to function as an inhibitor for GTPases is not hampered in the presence of Vav1. Whereas its ability to remove Ly-GDI from the target GTPase can explain why WT Vav1, but not OncVav1, can overcome inhibition by Ly-GDI and not Rho-GDI, it does not explain the observed synergy of Vav1 and Ly-GDI on NFAT activity. One plausible scenario is that the association of Vav1 and Ly-GDI also influences other signaling pathways. The observed increase in PLCγ tyrosine phosphorylation in cells transfected with Vav1 and Ly-GDI as
compared with cells transfected with Vav1 alone (Fig. 7A) may be one example of this.

Additional signaling cascades might also be influenced by Ly-GDI. We demonstrate that Ly-GDI associates with the SH2 domain of the adapter protein Shc. Shc is tyrosine-phosphorylated by ZAP-70 upon TCR engagement, and it then forms complexes with Grb2 as well as with other proteins (66). Moreover, Shc was shown to be involved in activation of c-Raf and mitogen-activated protein kinase, and it is required for TCR-induced interleukin-2 production (67). Vav1 also binds multiple signaling proteins through its SH2, SH3, and proline-rich regions, including adapter molecules such as Grb2, Crk, and Shc (68, 69) and signaling proteins such as ZAP-70 (70) and SLP-76 (71, 72). The functional significance of the interaction of Vav1 with the adapter proteins is unknown as yet. However, the association of Vav1 with signaling molecules such as ZAP-70 and SLP-76 influences NFAT activity (73, 74). The fact that Ly-GDI and Vav1 associate with adapter proteins that form multicomplexes as well as with signaling proteins and of course with each other suggests that they might influence various signaling pathways in T cells, including stimulation of phosphatidylinositol 3-kinase and the Ras/mitogen-activated protein kinase pathway.

Ly-GDI and Rho-GDI are very similar in structure (59). In summary, these studies reveal a novel molecular mechanism involved in the response of T cells to TCR engagement, and it then forms complexes with Grb2 as well as with other proteins (66). Moreover, Shc was shown to be involved in activation of c-Raf and mitogen-activated protein kinase, and it is required for TCR-induced interleukin-2 production (67). Vav1 also binds multiple signaling proteins through its SH2, SH3, and proline-rich regions, including adapter molecules such as Grb2, Crk, and Shc (68, 69) and signaling proteins such as ZAP-70 (70) and SLP-76 (71, 72). The functional significance of the interaction of Vav1 with the adapter proteins is unknown as yet. However, the association of Vav1 with signaling molecules such as ZAP-70 and SLP-76 influences NFAT activity (73, 74). The fact that Ly-GDI and Vav1 associate with adapter proteins that form multicomplexes as well as with signaling proteins and of course with each other suggests that they might influence various signaling pathways in T cells, including stimulation of phosphatidylinositol 3-kinase and the Ras/mitogen-activated protein kinase pathway.

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