Translocation of the SOS and Vav GDP/GTP exchange factors proximal to Ras and Rac GTPases localized in the plasma membrane glycolipid-enriched microdomains is a pivotal step required for T cell antigen receptor-induced T cell activation. Here we demonstrate that the T cell antigen receptor ζ-chain-associated ZAP-70 kinase and T cell antigen receptor ζ-chain immunoreceptor tyrosine-based activation motifs are essential for the membrane recruitment of SOS and Vav. Plasma membrane targeting of SOS or Vav begins with the assembly of ZAP-70 with Grb-2 and SOS. The subsequent tyrosine phosphorylation of LAT (linker for activation of T cell) by ZAP-70 leads to a shift in equilibrium from the ZAP-70-Grb-2-SOS(Vav) complex to the (Vav)SOS-Grb-2-LAT complex. This shift results in the targeting of SOS and Vav into glycolipid-enriched microdomains and initiation of the Ras and Rac signaling cascades involved in T cell activation, proliferation, and cytokine production.

The small GTPase Ras plays an important role in T cell signaling by promoting activation of the mitogen-activated protein kinase ERK by the Ras-Raf-MEK-ERK cascade (1). Other small GTP-binding proteins, such as Rac, Cdc42, and Rho, may regulate T cell antigen receptor (TCR)- and CD28-mediated catalytic activities of multiple downstream effectors, including the c-Jun N-terminal kinases (2, 9) and p38 mitogen-activated protein kinases (4–6). Conversion of GDP-bound GTPases to the active GTP-bound state by GDP/GTP exchange factors is critical for TCR-induced T cell activation, interleukin-2 production, and thymocyte development (7, 8). The hematopoietic lineage-specific Vav and more ubiquitously expressed SOS function as GDP/GTP exchange factors for Rho-related proteins, including Rac (9, 10), Cdc42, RhoA (11), and Ras (12), respectively. In activated T cells, SOS promotes the conversion of Ras to the GTP-bound active state (13), whereas Vav is involved in Rac GDP/GTP exchange (14, 15). Disruption of either the TCR or CD28 signaling pathways in Vav-deficient mice markedly decreases the amount of interleukin-2 secretion, maturation, and TCR-mediated cytoskeletal reorganization in T cells (16, 17). These Vav-deficient mice also display defects in TCR-induced intracellular calcium fluxes as well as in the activation of mitogen-activated protein kinase and the NF-κB transcription factor (18).

Recruitment of SOS proximal to Ras in the plasma membrane is an essential step for Ras activation in T cells (13, 19), and plasma membrane-targeted SOS derivatives activate components of the Ras signaling pathway, particularly the ERK kinase and AP-1 transcription factor (20). Previously, we (7) and others (8, 21) reported that a block in Ras activation occurs in anergic T cells. More recently, we showed that this Ras block is mediated by the impaired membrane translocation of SOS (22). This result prompted us to further investigate the mechanisms of recruitment of SOS, as well as Vav, to GTPase-containing signaling complexes in the plasma membrane of T cells.

SOS and Vav activation and their membrane targeting may be controlled by the adaptor protein Grb-2, which binds to SOS and Vav (13, 23). These Grb-2/SOS and Grb-2/Vav interactions involve the association of the Src homology 3 (SH3) domain of Grb-2 with proline-rich regions of SOS and Vav (13, 23). Considerably less is known about phosphoproteins that form docking sites for adaptor proteins, such as Grb-2, and mediate the recruitment of “SOS(Vav)+adaptor protein” complexes to the plasma membrane in an activated T cell. Upon TCR ligation, Grb-2 forms a complex with a pp36–38 tyrosine phosphoprotein (13, 24) recently identified as LAT (linker for activation of T cells) (25). LAT is localized primarily in plasma membrane glycolipid-enriched microdomains (GEM) of T cells (13, 24, 26) and is a substrate for the TCR ζ-chain (TCRζ)-associated ZAP-70 protein-tyrosine kinase (25). Consistent with its membrane localization, LAT may function as a central adaptor that recruits multiple proteins required for downstream signaling (25, 27–29). Upon TCR stimulation, ZAP-70 is translocated to the plasma membrane, and activated Lck enhances the plasma membrane accumulation of ZAP-70 (30). Immunoreceptor tyrosine-based activation motifs (ITAMs) of TCR-associated CD3 subunit phosphoproteins, which recruit ZAP-70 from the cytoplasm to the membrane-associated TCR-CD3 complex (31–33), may also form docking sites for the binding of secondary signaling proteins containing SH2 domains, including Grb-2 (32, 34). Conceivably, Lck- and ZAP-70-dependent phosphorylation of LAT may play a critical role in the assembly of LAT-Grb-2-containing GEM-associated signaling complexes proximal to TCRζ and downstream effectors, such as GEM-associated Ras and Rho/Rac.

In this study, we analyzed the roles of ZAP-70 and LAT in the recruitment of SOS, Vav, and Grb-2 from the cytoplasm to...
mature T cell enrichment columns (R & D Systems, Minneapolis, MN) 
(purity ≥95%). If not otherwise indicated, mouse T cells were stimulated 
(3 min, 37 °C) with 1 µg/10^6 cells of the biotin-conjugated 
anti-CD3 mAb either alone or together with the biotin-conju-
gated anti-CD4 mAb. Cross-linking of mAbs was accomplished using a 
biotinylated (Sigma) at a 4:1 ratio for 30 min at 4 °C followed by 
 incubation with 1% Triton X-100 plus 0.2% Nonidet P-40 (Nonidet P-40) or 60 m
 o-nitro-β-D-galactopyranoside (Sigma) supplemented with a mixture 
of protease and phosphatase inhibitors (22).

Subcellular Fractionation—Cells were lysed by sonication in ice-cold 
10 mM Tris, pH 7.4, 10 mM KCl, 1.5 mM MgCl_2, 2 mM EGTA hypotonic 
buffer containing the above described mixture of protease and phospha-
tase inhibitors (buffer A) (34). Lysates were centrifuged to remove 
nuclei and debris, and particulate membrane-containing and soluble 
 cytoplasm-containing fractions were separated by differential centrifu-
gation for 30 min at 100,000 × g. Membrane fractions were washed with 
ice-cold buffer A, solubilized by sonication in buffer A supplemented 
with 150 mM NaCl and either 1% Triton X-100 plus 0.2% Nonidet P-40 
or 1% Nonidet P-40, and centrifuged. Analysis of proteins in mem-
brane-bound fractions was performed after normalization for protein concentra-
tion levels.

Immunoprecipitation of Cellular Proteins and in Vitro Binding As-
says—If not otherwise indicated, precleared postnuclear cell lysates 
were normalized for protein concentration levels and immunoprecipi-
ted (3 h, 4 °C) with the specific polyclonal Abs or control isotype-
matched preimmune Ig precomplexed to 25 µl of protein A-Sepharose 
CL-4B (Amersham Pharmacia Biotech, Baie d'Urfe, Canada). This was 
followed by four washes of the precipitates with ice-cold lysis buffer. 
When membrane proteins were precipitated, the amount of an Ab used 
was empirically determined to quantitatively precipitate the amount 
of antigen available. In vitro binding assays were performed after cell 
disruption in lysis buffer containing 1% Nonidet P-40 plus 1% SDS. Cell 
lysates were boiled for 2 min, diluted 20-fold with lysis buffer contain-
ing 0.5% Nonidet P-40, and incubated with the fusion proteins immobi-
лизованными на агарозовых зернах.

Gel Electrophoresis and Immunoblotting—Precipitated proteins were 
solubilized in 2× Laemmli sample buffer, resolved by SDS-PAGE 
(8–16% gradient gel, Novex, San Diego, CA) under reducing conditions, 
transferred to nitrocellulose membranes, and immunoblotted with the indicated Abs (22). Signal intensities were 
quantified using a Molecular Image System and Molecular Analyst 
imaging software (Bio-Rad).

In Vivo Incorporation of [32P]Labeled Guanine Nucleotides by Ras—T 
cells (10^7) were cultured for 4 h in phosphate-free RPMI medium con-
taining dialyzed 10% heat-inactivated fetal bovine serum and then 
labeled with [32P]ATP (15 µCi; NEN Life Science Products) in 25 µl of 
phosphate-free RPMI/HEPES (2 mM). Precleared postnuclear lysates 
were immunoprecipitated by protein G-agarose precoated to anti-Ras (10 
µg) mAb, and [32P]labeled guanine nucleotides bound to Ras were frac-
tionated using polyethyleneimine thin layer chromatography plates 
(J. T. Baker Inc.) as described (19).

Kinase Assays—T cells were incubated for 3–4 h at 37 °C in fetal 
bovine serum-free RPMI 1640 medium before stimulation to reduce 
background kinase activities to workable levels. Proteins immunopre-
cipitated from precleared postnuclear lysates were assayed for associ-
ated in vitro kinase activity after washing the beads in kinase buffer (25 
mM HEPES, pH 7.4, 5 mM MgCl_2) by incubation (30 min, 30 °C) with 
[γ-32P]ATP (15 µCi; NEN Life Science Products) in 25 µl of kinase 
buffer containing 3 µg of myelin basic protein (Upstate Biotechnology 
Inc.) as substrate. Reactions were stopped by boiling with gel sample 
buffer. Myelin basic protein was resolved by SDS-PAGE, and its 
phosphorylation was visualized using a phosphor imager (Bio-Rad). Immu-
noblotting showed that equal amounts of the ERK-1 and PAK proteins 
were precipitated before and after stimulation of all Jurkat T cell 
variants.

GEM Fractionation—Purification of the GEM fraction was per-
formed as described (26). Cells were lysed by brief sonication in ice-cold 
25 mM MES, pH 6.5, 150 mM NaCl, 5 mM EDTA lysis buffer containing 
1% Triton X-100 and supplemented with a mixture of protease and 
phosphatase inhibitors (22). Lysates were mixed with an equal volume of 
80% sucrose made in lysis buffer and were overlaid with 2 ml of 30% 
sucrose and 1 ml of 5% sucrose. GEM-enriched fractions were collected 
and quantified using a Molecular Imager System and Molecular Analyst 
imaging software (Bio-Rad).
TCR-activated Jurkat T cells treated with 60 mM tyrosine (anti-PY), anti-ZAP-70, anti-Vav, anti-Grb-2, or anti-SOS mAb. anti-PY immunoprecipitates from Triton X-100 lysates of resting or TCR-stimulated Jurkat T cells were immunoblotted with an anti-phospho-Ig (C-Ig), equal amounts of precipitating Abs were used, and the relative signal intensities (RSI) ((density × area)/lane) of phospho-LAT in SOS, Vav, and Grb-2 immunoprecipitates were expressed as a/b × 100, where a is the specific signal intensity of a particular phospho-LAT band, and b is the signal intensity of phospho-LAT in Grb-2 immunoprecipitates from TCR-activated Jurkat T cells. D, Grb-2 mediates the association of SOS with ZAP-70 and LAT in T cells. SOS immunoprecipitates from resting or TCR-stimulated mock- or Grb-2-depleted Jurkat T cell lysates were immunoblotted with anti-ZAP-70, anti-Grb-2, anti-LAT, and anti-SOS Abs. Grb-2 depletion was performed by three sequential rounds of immunoprecipitation with an anti-Grb-2 Ab, and its efficiency was confirmed by anti-Grb-2 immunoblotting (data not shown). The results shown are representative of one of three separate reproducible experiments of each type.

RESULTS

TCR Ligation Stimulates the Association of ZAP-70 with Grb-2, SOS, and Vav—We investigated how SOS and Vav are recruited to the plasma membrane. Initially, we identified those proteins that interact with SOS, Vav, and Grb2 after TCR ligation in Jurkat T cells. As reported (13, 24, 25, 34), TCR cross-linking induced the association of Grb-2 with tyrosine-phosphorylated LAT (Fig. 1A). Phospho-LAT was detected in SOS but not Vav immunoprecipitates of TCR-stimulated Jurkat T cell lysates. Interestingly, ZAP-70 was found to interact with SOS, Vav, and Grb2 in unstimulated T cells, and these associations were significantly enhanced after TCR stimulation. These observations demonstrate for the first time that ZAP-70 interacts with SOS in T cells. Moreover, Grb-2, SOS, and Vav were present in immunoprecipitates of ZAP-70 (data not shown). TCR ligation also stimulated the association of SOS and Vav with Grb2 as well as the interaction of SOS and Vav.

The relative stoichiometry of interaction induced by TCR ligation between phospho-LAT and SOS was about 10-fold less than that between phospho-LAT and Grb2, despite the use of equal amounts of anti-SOS and anti-Grb2 Abs for immunoprecipitation (Fig. 1A). By comparison, a similar relative stoichiometry of interaction between ZAP-70 and either SOS, Vav, or Grb-2 was observed. Most phospho-LAT molecules are palmitoylated and are localized in Triton X-100-insoluble GEM (26). Partitioning of LAT to GEM may therefore diminish the co-precipitation of phospho-LAT with SOS and Vav when these membrane proteins are solubilized in Triton X-100. This notion was supported by the finding that solubilization of these proteins in n-octyl-β-D-glucopyranoside, a nonionic detergent that dissociates GEM while preserving protein-protein interactions, significantly increased the co-precipitation of phospho-LAT with both SOS and Vav (Fig. 1B). This result suggests that TCR cross-linking induces the partitioning of a significant proportion of SOS and Vav to LAT-containing GEM. In addition, T cell lysis by n-octyl-β-D-glucopyranoside preserves and does not further enhance Grb-2/ZAP-70, SOS/ZAP-70, and SOS/Vav associations (Fig. 1C), indicating that these interactions are mostly GEM-independent.

We next analyzed whether the SOS/ZAP-70 interaction in T cells depends on the presence of Grb-2. SOS was immunoprecipitated from T cell lysates after several rounds of immunodepletion of Grb-2 with an anti-Grb-2 Ab. The high efficiency of Grb-2 immunodepletion was confirmed by immunoblotting of depleted cell lysates with anti-Grb2 (data not shown). If such interaction depends on the presence of Grb-2, immunodepletion of Grb-2, which bridges the binding of SOS with ZAP-70 or LAT, would be expected to remove ZAP-70 and LAT from SOS immunoprecipitates. Fig. 1D shows that TCR stimulation significantly increased the amounts of ZAP-70 and LAT that co-immunoprecipitated with SOS in nondepleted T cell lysates. Conversely, sequential Grb-2 immunodepletion rendered the SOS immunoprecipitates virtually devoid of detectable ZAP-70, LAT, and Grb-2. These findings are consistent with the formation of TCR-induced trimeric SOS-Grb-2-LAT complexes and, more importantly, provide the first demonstration of SOS/Grb-2/ZAP-70 interactions in TCR-stimulated T cells.

Inducible Association of ZAP-70 with SOS and Grb-2 Is Phosphotyrosine- and Lck-dependent—The requirement of TCR-induced tyrosine phosphorylation for the association of ZAP-70 with Grb-2-SOS complexes was analyzed. We deter-
of the Lck-deficient JCaM1.6 T cells demonstrated that Lck, which is required for tyrosine phosphorylation of ZAP-70 and LAT (25, 31), is essential for the inducible binding of ZAP-70 and LAT to Grb-2 in the plasma membrane of Jurkat T cells (Fig. 2B). This result, coupled with the induced formation of Grb-2-ZAP-70 and SOS-ZAP-70 complexes, strongly suggests that a phosphotyrosine-dependent mechanism controls the binding of ZAP-70 to Grb-2 and SOS in T cells.

Structural Requirements for the Assembly of the ZAP-70-Grb-2-SOS Complex in T Cells—We next determined how the ZAP-70-Grb-2-SOS complex is formed. Since ZAP-70 does not possess an SH3 domain and SOS is not tyrosine-phosphorylated upon TCR stimulation, we considered the possibility that phospho-ZAP-70 interacts indirectly with SOS through the SH2 domain of Grb-2. The ability of an immobilized GST-Grb-2 fusion protein to pull down ZAP-70 from TCR stimulation T cells primary T cell lysates was determined after the lysates were boiled in SDS-containing lysis buffer to disrupt any preexisting associations. Wild-type full-length GST-Grb-2 and GST-Grb-2 SH2 domain fusion proteins precipitated significant amounts of ZAP-70 from TCR/CD4-activated C57BL/6J spleen T cells (Fig. 2C). Negligible binding of ZAP-70 to the GST-Grb-2 mutant containing the SH2 domain loss-of-binding mutation (R86K) was observed. Conversely, the NH2-terminal SH3 domain (P49L) and COOH-terminal SH3 domain (G203R) mutations did not alter the binding of ZAP-70 to immobilized GST-Grb-2. The binding of ZAP-70, LAT, and Vav to the Grb-2-SH2 domain was also evident in a pull-down assay of membrane-enriched fractions from unstimulated or TCR/CD4-activated C57BL/6J thymocytes solubilized by sonication in 1% Nonidet P-40 buffer under non-denaturing conditions (in the absence of SDS) (Fig. 2D).

To further examine how ZAP-70 interacts with Grb-2, P116 ZAP-70-deficient T cells were transiently transfected with the pSX vector expressing either wild-type ZAP-70 (ZAP-70:WT) or the ZAP-70 Y493F mutant (ZAP-70:Y493F). ZAP-70 is activated by the Lck-mediated tyrosine phosphorylation of its residue Tyr-493, which in turn stimulates the phosphorylation of residues Tyr-292 and Tyr-492, two additional major sites of ZAP-70 phosphorylation (37, 41). As shown in Fig. 2E (upper panel), TCR cross-linking significantly enhanced the tyrosine phosphorylation of ZAP-70:WT but not ZAP-70:Y493F, consistent with an essential role for Tyr-493 in the tyrosine phosphorylation of ZAP-70:WT and Tyr-492, two additional major sites of ZAP-70 phosphorylation (37, 41). In agreement with the results presented above, ZAP-70 (70

![Biochemical analysis of ZAP-70/Grb-2/SOS(Vav) interactions.](http://www.jbc.org/)
ZAP-70 Regulates the Membrane Targeting of SOS and Vav

**Fig. 3.** Analysis of TCR-induced SOS-, Vav-, and Grb-2-containing signaling complexes in the plasma membrane of wild-type and P116 Jurkat T cells. Particulate membrane-containing fractions of unstimulated or TCR-stimulated Jurkat T cells were normalized for protein concentration levels and then immunoprecipitated with an anti-SOS (A), anti-Vav (B), or anti-Grb-2 (C) Ab. The amount of an Ab used was empirically determined to quantitatively precipitate the amount of antigen available. Resulting protein complexes were resolved by SDS-PAGE and immunoblotted with an anti-phosphotyrosine (anti-p-Tyr) mAb (lower panels). The blots were stripped and reprobed with an anti-Vav, anti-Grb-2, anti-SOS, anti-ZAP-70, anti-LAT, anti-SLP-76, or anti-CD3ε mAb (upper panels). Molecular weight (Mw) markers and the position of migration of IgH and IgL chains are indicated. The results shown are representative of one of three reproducible experiments.

| Fraction | Jurkat | P116 |
|----------|--------|------|
| TCR      | none TCR | none TCR |
| IB anti-SOS | | |
| IB anti-Grb-2 | | |
| IB anti-Grb-2 | | |
| IB anti-Vav | | |
| IB anti-LAT | | |
| IB anti-ZAP-70 | | |
| IB anti-CD3ε | | |

| Fraction | Jurkat | P116 |
|----------|--------|------|
| TCR      | none TCR | none TCR |
| IB anti-SOS | | |
| IB anti-Grb-2 | | |
| IB anti-ZAP-70 | | |
| IB anti-CD3ε | | |

kDa) and Vav (95 kDa) are among the most prominent tyrosine phosphoproteins associated with SOS in the membrane of wild-type Jurkat T cells (Fig. 3A, bottom). The identity of the SOS-associated 62-kDa phosphoprotein is presently unknown and does not appear to be the membrane-localized RasGAP-associated phosphoprotein p62δk (42). TCR cross-linking induced the tyrosine phosphorylation of membrane-bound Vav and its association with the ZAP-70 and CD3ε phosphoproteins as well as Grb-2 and SOS in wild-type Jurkat T cells (Fig. 3B). In contrast, the extent of TCR-induced tyrosine phosphorylation of Vav and the amounts of Vav-associated proteins were reduced significantly in ZAP-70-deficient P116 T cells. The association of SOS with ZAP-70 and Vav is relatively specific, given the absence of other protein-tyrosine kinases (e.g. Fyn and Lck) and GDP/GTP exchange factors (e.g. C3G) in the SOS and Vav immunoprecipitates.2 In addition, LAT was found in SOS and Vav immunoprecipitates from plasma membrane fractions solubilized in n-octyl-β-D-glucopyranoside, which dissociates GEM (Fig. 3, A and B). Note also that while TCR stimulation significantly increased the association of SOS and Vav with Grb-2 in the membrane of wild-type Jurkat T cells (Fig. 3, A and B), such complexes were absent from membrane fractions of ZAP-70-deficient P116 T cells. Taken together, these data indicate that ZAP-70 is essential for the membrane targeting of SOS and Vav.

ZAP-70 Is Essential for the Assembly of Grb-2-SOS(Vav), Grb-2-LAT, and Grb-2-TCRζ Membrane Complexes—Analyses of membrane-associated Grb-2 illustrated that Grb-2 is constitutively present in similar amounts in membrane fractions obtained from both resting and TCR-activated wild-type and ZAP-70-deficient Jurkat T cells (Fig. 3C). Thus, the impaired membrane recruitment of SOS and Vav in ZAP-70-deficient Jurkat T cells does not result from either the reduced ability or failure of Grb-2 to be recruited to the plasma membrane.

TCR stimulation induced the co-precipitation of membrane-bound Grb-2 with many phosphoproteins, including ZAP-70, LAT, the TCR-CD3 complex and SLP-76 (Fig. 3C). ZAP-70 was essential for optimal tyrosine phosphorylation of Grb-2-associated LAT, SLP-76, and Vav. Importantly, TCR stimulation effectively induced the recruitment of SOS and Vav to membrane-bound Grb-2 in wild-type but not ZAP-70-deficient Jurkat T cells (Fig. 3C). In addition, although TCR stimulation enhanced the tyrosine phosphorylation and amount of the Grb-2-associated TCR-CD3 complex in wild-type Jurkat T cells, this was not evident in ZAP-70-deficient Jurkat T cells. The latter finding confirms the association of Grb-2 with TCRζ in the plasma membrane (34) and suggests that ZAP-70 controls the basal and TCR-induced association of Grb-2 with phosphorylated CD3ε components of the TCR complex. These observations provide direct evidence for an essential role for ZAP-70 in the assembly of Grb-2-SOS(Vav), Grb-2-TCRζ, and LAT-Grb-2 membrane complexes in TCR-stimulated T cells.

**LAT Influences the Recruitment of SOS and Vav to GEM in the Plasma Membrane—**LAT-deficient J.CaM2 T cells are defective in several TCR-mediated signaling events downstream of TCRζ and ZAP-70, such as ERK-1 activation, Vav tyrosine phosphorylation, calcium-dependent signaling, and interleukin-2 gene expression (27). Nonetheless, the role of LAT in the plasma membrane targeting of SOS and Vav is not presently known. It is possible that, in addition to the assembly with Grb-2, SOS, and Vav, ZAP-70 promotes the plasma membrane targeting of SOS and Vav by phosphorylating LAT, a downstream effector of ZAP-70. Consistent with this hypothesis, we found that J.CaM2 T cells are defective in the TCR-induced membrane targeting of Vav (Fig. 4A). In contrast, the presence of LAT is not absolutely required for optimal plasma membrane targeting of SOS. Furthermore, similar amounts of Grb-2 were present in the plasma membranes of Jurkat and J.CaM2 T cells (data not shown).

To further understand the structural basis of interaction of LAT with SOS and Vav, we analyzed TCR-dependent signaling in J.CaM2 T cells stably transfected with Myc epitope-tagged wild-type LAT (LAT-WT), LAT:Y171F/Y191F, or LAT:C26A/C29A (26). The use of J.CaM2 T cells minimized any potential contribution of endogenous LAT to downstream signaling. Overexpression of the LAT:Y171F/Y191F mutant in wild-type

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2 K. V. Saldin, unpublished observations.
Jurkat T cells markedly reduces the binding of Grb-2 to LAT and blocks the transcriptional activity of NFAT and AP-1 (25). Mutation of LAT Cys-26 and Cys-29 blocks the palmitoylation, translocation to GEM, and TCR-induced tyrosine phosphorylation of LAT (26). Fig. 4B shows that the expression of LAT:WT, LAT:C26A/C29A, and LAT:Y171F/Y191F migrate differently on SDS-PAGE. LAT:WT and LAT:C26A/C29A each migrate as two bands with molecular mass of 36 and 40 kDa. The slower migrating LAT:C26A/C29A migrated mainly as a 40-kDa band. D. GEM-enriched fractions from resting (D and E) or TCR-stimulated (E) LAT-deficient J.CaM2 transfectants were prepared by differential ultracentrifugation in a 5%/30%/40% gradient. GEM-enriched fractions were analyzed by SDS-PAGE and immunoblotted with anti-LAT Ab. The results shown are representative of one of three reproducible experiments.

The fact that LAT palmitoylation at Cys-26 and Cys-29 is essential for the interaction of phospho-LAT with SOS, Vav, ZAP-70, and Grb, and Tyr-171 and Tyr-191 are required for phospho-LAT to associate with Grb-2 and SOS. The fact that LAT palmitoylation at Cys-26 and Cys-29 is essential for the interaction of phospho-LAT with SOS, Vav, ZAP-70, and Grb, and Tyr-171 and Tyr-191 are required for phospho-LAT to associate with Grb-2 and SOS.

Both ZAP-70 and LAT Are Required for ERK-1 and PAK Activation—We addressed the question of whether ZAP-70-dependent and LAT-mediated recruitment of SOS and Vav into GEM is required for the activation of SOS and Vav downstream effectors, ERK-1 and PAK, respectively. ERK-1 func-
FIG. 5. ZAP-70 and LAT regulate the activities of SOS and Vav downstream effectors, including ERK-1 and PAK. A, kinase activities associated with ERK-1 and PAK immunoprecipitates were analyzed in unstimulated or TCR-stimulated wild-type, ZAP-70-deficient P116 or LAT-deficient J.Cam2 T cells stably transfected with the LAT:WT, LAT:C26A/C29A, or control empty vectors using myelin basic protein as substrate. B, kinetics of TCR-induced SOS-mediated Ras GDP/GTP exchange in TCR-stimulated wild type and P116 Jurkat T cells. In vivo incorporation of 32P-labeled guanine nucleotides by Ras in wild type and P116 Jurkat T cells was assayed by polyethyleneimine thin layer chromatography. C and D, LAT-independent interaction of ZAP-70 with Grb-2, SOS, and Vav. Cell lysates isolated from unstimulated or TCR-stimulated Jurkat, J.CaM2, and P116 T cells were incubated with GST fusion protein (10 μg) encoding full-length Grb-2 immobilized on agarose beads or anti-SOS (C) and anti-Vav (D) Abs. Precipitates were analyzed by SDS-PAGE and immunoblotting with anti-ZAP-70, anti-phosphotyrosine (anti-PY), and anti-Vav mAbs. The results shown are representative of one of three separate reproducible experiments of each type.

lations downstream of Ras in the SOS-Ras-Raf-ERK pathway (19), and PAK controls the activation of c-Jun N-terminal kinase and p38 mitogen-activated protein kinase as well as cytoskeleton organization downstream of the Vav-Rac pathway (2, 4, 43). Fig. 5A shows that TCR-induced ERK-1 and PAK kinase activities were virtually undetectable in both ZAP-70-deficient P116 and LAT-deficient J.Cam2 T cells relative to that of wild type Jurkat T cells. Stable transfection of LAT:WT, but not LAT:C26A/C29A, into J.Cam2 T cells restored ERK-1 and PAK activation. In addition, analysis of the [32P]orthophosphate-labeled guanine nucleotides bound to Ras revealed that TCR stimulation enhances GDP/GTP exchange on Ras in wild-type but not ZAP-70-deficient Jurkat T cells (Fig. 5B). Thus, ZAP-70 and LAT are required to regulate the TCR-induced SOS-Ras-Raf-ERK and Vav-Rac-Pak c-Jun N-terminal kinase (p38 mitogen-activated protein kinase) signaling pathways. Moreover, failure of LAT:C26A/C29A-transfected J.Cam2 T cells to up-regulate ERK-1 and PAK activities upon TCR stimulation demonstrates that Cys-26- and Cys-29-dependent partitioning of LAT into GEM is required for activation of ERK-1 and PAK.

Interactions of ZAP-70 with Grb-2, SOS, and Vav Occur Independently of LAT—Consistent with the function of LAT downstream of ZAP-70, we found that the binding of ZAP-70 to GST-Grb-2 as well as SOS and Vav was unaltered in unstimulated and TCR-activated J.Cam2 T cells (Fig. 5C and D). Basal and TCR-induced association of Vav with SOS were unimpaired in the absence of LAT. In contrast, in parallel to the down-regulation of TCR-induced tyrosine phosphorylation of Vav in P116 and J.Cam2 cells (27), the binding of Vav to GST-Grb-2 was significantly diminished in lysates from both LAT- and ZAP-70-deficient T cells. These results demonstrate that ZAP-70 interacts with Grb-2, SOS, and Vav upstream of LAT and suggest that there is another level of regulation of the formation of SOS- and Vav-dependent multimeric complexes. This regulation is mediated by the assembly of ZAP-70-Grb2-SOS-Vav complexes upstream of LAT.

Membrane Translocation of SOS and Vav Requires TCRζ ITAMs—To test the possibility that TCRζ ITAMs function as docking sites for a ZAP-70-Grb2-SOS(Vav) signaling complex, we assayed the TCR-induced membrane translocation of SOS and Vav in T cells from TCRζζ/mice reconstituted with the TCRζζ transgene encoding TCRζζ molecules in which all three ITAMs were deleted (TCRζζ-D67−150-Tg) (35). Although this deletion results in a signaling-deficient TCRζζ that is unable to recruit ZAP-70 to the TCR-Cd3 complex, TCRζζ-D67−150-Tg promotes TCR surface expression and T cell maturation (35). We found that TCR cross-linking enhances the translocation of SOS and Vav to the plasma membrane in control C57Bl/6 but not TCRζζ-D67−150-Tg thymocytes (Fig. 6A). Similar to that found in human Jurkat T cells, immunoblotting with anti-ZAP-70 and anti-Vav mAbs demonstrated that membrane-associated SOS binds ZAP-70 and Vav following TCR stimulation in mouse thymocytes. By comparison, significantly lower amounts of ZAP-70 were associated with membrane-bound SOS and Vav in TCRζζ-D67−150-Tg mice due to the impaired recruitment of SOS and Vav to the plasma membrane. The association of SOS and Vav was also noticeably decreased in TCR-stimulated TCRζζ-D67−150-Tg thymocytes. Note that the impaired translocation of SOS was accompanied by increased amounts of SOS and Vav and ZAP-70 in the cytoplasm of the TCRζζ-D67−150-Tg mice (Fig. 6B). In contrast, the amounts of membrane-associated Grb-2 in unstimulated and stimulated C57Bl/6 and TCRζζ-D67−150-Tg thymocytes were comparable (Fig. 6C). These data suggest that deficient membrane recruitment of SOS and Vav to the TCR complex

This regulation is mediated by the assembly of ZAP-70-Grb2-SOS-Vav complexes upstream of LAT.
does not result from the decreased translocation of Grb-2 to the plasma membrane but rather reflects the impaired recruitment of ZAP-70 to the TCRζ ITAMs. Consistent with this notion, translocation of ZAP-70 to the plasma membrane is deficient in TCRζ-D67–150-Tg thymocytes (Fig. 6C).

**DISCUSSION**

A protein-tyrosine kinase-dependent mode of translocation of the SOS and Vav GDP/GTP exchange factors proximal to Ras and Rac GTPase-containing signaling complexes in the plasma membrane is known to be a pivotal step required for TCR-induced T cell activation and function. However, the mechanism by which this translocation occurs was not previously elucidated. The observation that ZAP-70 is recruited to the plasma membrane upon TCR stimulation and that activated Lck enhances the plasma membrane accumulation of ZAP-70 (30) raises the question of whether ZAP-70 influences the plasma membrane targeting of ZAP-70 downstream effector proteins that function as a scaffold and recruit SH2 domain-containing signaling proteins (32). In this study, we investigated the role of ZAP-70, LAT and their associated signaling proteins in the translocation of SOS and Vav from the cytoplasm to the plasma membrane after TCR ligation. Using ZAP-70-deficient Jurkat T cells, we found that ZAP-70 is essential for the constitutive and TCR-induced membrane targeting of SOS and Vav as well as the formation of SOSGrb-2, VavGrb-2, SOS-Vav, and SOS(Vav)ZAP-70 complexes in the plasma membrane. Our data indicate that ZAP-70 controls the membrane translocation of SOS and Vav by 1) binding directly to the Grb-2 SH2 domain in Grb-2SOS and Grb-2Vav complexes; 2) increasing the tyrosine phosphorylation of Vav, which promotes the association of Vav with ZAP-70, Grb-2, and SOS in the plasma membrane; and 3) enhancing the tyrosine phosphorylation of membrane-localized LAT and its association with Grb-2SOS and Grb-2Vav complexes.

The use of LAT-deficient J.CaM2 T cells revealed that LAT is essential for the TCR-induced recruitment of SOS and Vav into GEM. This observation provides a biochemical basis for the rapidly emerging concept of sphingolipid-cholesterol-rich microdomains or GEM that cluster critical signaling molecules, including Ras and Rac GTPases, in the plasma membrane of activated T cells (26, 28, 29).

Our results favor a model in which the plasma membrane targeting of SOS or Vav begins with the assembly of ZAP-70 with Grb-2, SOS, and Vav, which occurs independently of the subsequent tyrosine phosphorylation of LAT (Fig. 7). According to this model, upon TCR stimulation ZAP-70 is tyrosine-phosphorylated by Lck, translocated to the plasma membrane to associate with TCRζ (step 1), and by functioning as a docking phosphoprotein potentiates the formation of a complex between SOS and Vav with Grb-2 (steps 2 and 3). Support for such a role for ZAP-70 is also provided by the finding that a membrane-targeted CD2/ZAP-70 chimera induces late signaling events in the absence of TCR stimulation but in the presence of a functional kinase-active Lck (44). Subsequent tyrosine phosphorylation...
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In conclusion, our results demonstrate a pivotal role of ZAP-70 in TCR-induced translocation of SOS and Vav to the plasma membrane and GEM in T cells. ZAP-70 is critical for the recruitment of SOS and Vav to the plasma membrane and regulates the activation of the mitogen-activated protein kinase and PKA signaling cascades involved in T cell activation, proliferation, and cytokine production.

Acknowledgments—We sincerely thank A. Weiss, R. T. Abraham, P. E. Love, L. E. Samelson, E. W. Shores, G. A. Koretzky, D. Motto, and M. Matsuda for the kind gifts of reagents; all members of our laboratory for valuable advice and encouragement; and Dr. L. E. Samelson for critical evaluation of the manuscript. We also thank Anne Leaist for expert assistance with the preparation of the manuscript.

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J. Biol. Chem. 2000, 275:5966-5975.
doi: 10.1074/jbc.275.8.5966

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