Stimulation of the T cell antigen receptor (TCR) induces tyrosine phosphorylation of numerous intracellular proteins. We have recently investigated the role of the adaptor protein Shb in the early events of T cell signaling and observed that Shb associates with Grb2, linker for activation of T cells (LAT) and the TCR ζ-chain in Jurkat cells. We now report that Shb also associates with phospholipase C-γ1 (PLC-γ1) in these cells. Overexpression of Src homology 2 domain defective Shb caused diminished phosphorylation of LAT and consequently the activation of mitogen-activated protein kinases was decreased upon TCR stimulation. In addition, the Shb mutant also blocked phosphorylation of PLC-γ1 and the increase in cytoplasmic Ca²⁺ following TCR stimulation. Nuclear factor for activation of T cells is a major target for Ras and calcium signaling pathways in T cells following TCR stimulation, and the overexpression of the mutant Shb prevented TCR-dependent activation of the nuclear factor for activation of T cells. Consequently, endogenous interleukin-2 production was decreased under these conditions. The results indicate a role for Shb as a link between the TCR and downstream signaling events involving LAT and PLC-γ1 and resulting in the activation of transcription of the interleukin-2 gene.

The T cell response is initiated by the presentation of an antigen to the T cell receptor (TCR). This is followed by rapid phosphorylation of tyrosines on both the receptor itself and cytoplasmic proteins, the initiation of several signaling cascades, and subsequently the activation of interleukin-2 (IL-2) gene transcription and other T cell immune functions. The early events in T cell signaling involve the activation of several protein tyrosine kinases. After TCR ζ-chain phosphorylation (by the protein tyrosine kinases Lck or Fyn), a Syk family protein tyrosine kinase, ZAP70, binds the ζ chain immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex (1) and consequently becomes phosphorylated and activated. Active ZAP70 is then responsible for phosphorylation and activation of many additional substrates: for example, phospholipase C-γ1 (PLC-γ1), p36/38 linker for activation of T cells (LAT) (2), the guanine nucleotide exchange factor Vav, and the adaptor protein SLP-76 (3). In the course of these events, PLC-γ1, Grb2, and phosphoinositide 3-kinase all associate with tyrosine-phosphorylated p36/38 LAT (4–7). Phosphorylation of PLC-γ1 activates this enzyme, which then hydrolyzes phosphatidylinositol phosphate to yield diacylglycerol and inositol 1,4,5-tris-phosphate, the latter messenger mobilizing intracellular Ca²⁺. The resulting increase in cytoplasmic Ca²⁺ activates calmodulin, which mediates nuclear translocation and activation of the T cell-specific transcription factor nuclear factor for activation of T cells (NFAT) (8).

Activation of the Ras signaling pathway in response to TCR stimulation involves p36/38 LAT, Grb2 and Sos (5). As a consequence, mitogen-activated protein (MAP) kinases are stimulated, causing a subsequent nuclear translocation of the activating protein-1 (AP-1) and NFAT transcription factors (9, 10).

IL-2 gene expression is a common measure of T cell activation and requires the co-operation of several signaling pathways and different transcription factors, including AP-1, NFAT, Oct-1, and NF-κB (11). These transcription factors bind to the IL-2 gene regulatory elements and induce transcription.

Shb is an adaptor protein that contains a Src homology 2 (SH2) domain (12) in its C terminus, proline-rich sequences in its N terminus, and a central phosphotyrosine binding (PTB) domain (13). The proline-rich motifs in Shb have been found to interact with the SH3 domain of the p85 subunit of phosphoinositide 3-kinase, Src tyrosine kinase, and Epox (14), whereas the SH2 domain of Shb associates with the PDGF β-receptor and the FGF receptor-1 (14). In Jurkat T cells, Shb was found to interact with Grb2, p36/38, and the ζ-chain of the CD3 receptor (13). The association between Shb and Grb2 is mediated by proline-rich/SH3 domain interactions, whereas p36/38 binds to the Shb PTB domain in a phosphotyrosine-dependent manner. Shb interacts with the CD3-associated ζ-chain via its SH2 domain.

In this study we describe interactions between Shb and LAT and how a functional Shb SH2 domain is vital for phosphorylation of this Shb-LAT complex. In addition, we demonstrate that PLC-γ1 can interact with Shb and that a functional SH2 domain of Shb is important for PLC-γ1 activation and Ca²⁺ signaling. It is suggested that Shb plays a role in linking the TCR to cellular activation and IL-2 production in the immune response.
EXPERIMENTAL PROCEDURES

Peptides and Reagents—Monoclonal anti-phosphotyrosine 4G10 and anti-Nck/PLC-γ1 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-CD3 antibodies were from Becton Dickinson (San Jose, CA) and also kindly provided by Dr Hamid Band (Harvard Medical School, Boston, MA). R-PE-rat anti-human IL-2 antibodies were from Pharmingen (San Diego, CA). Antibodies recognizing conjugated antibodies (anti-mouse and anti-rabbit) and the ECL detection system were from Amersham Pharmacia Biotech. Protein A/agarose was from Pierce.

DNA Constructs—The Shb R522K plasmid was described previously (13). Briefly, the arginine at position 522 (which is necessary for phosphotyrosine binding ability of the SH2 domain) was converted into a methionine using site-directed mutagenesis, and cell extracts were immunoprecipitated with Shb, LAT, or buffer (0.15 M NaCl, 0.025 M Tris, pH 7.5, 1% Brij 96, 5 mM EDTA, 1 mM DTT) and subsequently expressed previously (2). PLC-δ1 antibodies were from Pharmingen (San Diego, CA). Antibodies recognizing conjugated antibodies (anti-mouse and anti-rabbit) and the ECL detection system were from Amersham Pharmacia Biotech. Protein A/agarose was from Pierce.

Binding Experiments—Jurkat T cells were maintained in RPMI medium supplemented with 10% FCS. Cells were collected by centrifugation and suspended in RPMI 1640 medium lacking serum before stimulation with the CD3 antibody at 37 °C for 2 min. The cells were pelleted and lysed in either Triton X-100 (0.15 M NaCl, 0.05 M Tris, pH 7.5, 0.5% Triton X-100, 1 mM NaF, 0.1 mM orthovanadate, 100 units/ml Trasylol, 2 mM phenylmethylsulfonyl fluoride) or Brij lysis buffer (0.15 M NaCl, 0.025 M Tris, pH 7.5, 1% Brij 96, 5 mM EDTA, 1 mM orthovanadate, 100 units/ml Trasylol, 2 mM phenylmethylsulfonyl fluoride) for 10 min. Nuclei were pelleted by centrifugation, and cell extracts were immunoprecipitated with Shb, LAT, or PLC-γ1 antibodies. The immune complexes were pelleted with 50 μl of protein A-Sepharose and subsequently washed three times with PBS, 1% Triton. The samples were then subjected to SDS-polyacrylamide gel electrophoresis in the absence or presence of 1% Triton X-100 in blocking solution (5% bovine serum albumin in PBS, 0.5% Tween 20) or 5% milk in PBS, 0.1% Tween (all other antibodies) and subsequently analyzed for tyrosine phosphorylation of Shb by immunoblotting with 4G10 anti-phosphotyrosine antibody. The positions of p55 Shb and p36/38 LAT are indicated.

Measurements of Intracellular IL-2 Levels—Cells were fixed in 4% paraformaldehyde and permeabilized according to the protocol “Immunofluorescent staining of intracellular cytokines for flow cytometric analysis” from Pharmingen. The samples were then stained with an R-PE-rat anti-human IL-2 antibody for 30 min in the dark, washed, and subsequently analyzed for fluorescence by flow cytometry on a FACSCalibur cell sorter from Becton Dickinson.

Measurements of Cytoplasmic Ca2+—Batches of 2.5 × 10^6 Jurkat-neo or Jurkat-R522K-2 cells were suspended in 5 ml of medium containing 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 1.3 mM CaCl2, 3 mM glucose, and 25 mM HEPES, with the pH adjusted to 7.4 with NaOH. After addition of 5 μl fura-2 acetoxymethylester the cells were allowed to accumulate fura-2 for 40 min at 37 °C. After loading, the cells were spun down and washed with identical medium lacking the indicator. After suspension in 1 ml of medium, the cells were transferred to a 1-cm quartz cuvette placed in the thermostatically controlled (37 °C) cuvette holder of a time-sharing multichannel spectrophotofluorometer (20). The cytoplasmic Ca2+ concentration was measured as described previously (21), compensating for leakage of the Ca2+ indicator fura-2 from the cells during the experiments but without initial compensation for extracellular indicator. Addition of anti-CD3 was made by injecting 10 μl of a 100-fold concentrated solution.

RESULTS

Shb Is Phosphorylated upon TCR Stimulation in Jurkat T Cells—We previously failed to detect tyrosine phosphorylation of Shb in response to TCR activation in Jurkat cells (13). It is conceivable, however, that tyrosine-phosphorylated Shb isoforms were concealed in those experiments by IgG background reactivity. We have subsequently observed tyrosine phosphorylation of Shb in response to FGF (22) and NGF (23) and thus decided to re-evaluate the possibility of TCR-dependent phosphorylation of Shb. Cell extracts from CD3-stimulated and unstimulated Jurkat cells were subjected to immunoprecipitation using the Shb antibody, under native conditions or after boiling for 2 min in the presence of 1% SDS. Electrophoresis was performed under nonreducing conditions to minimize the IgG background. Western blot analysis using a phosphotyrosine antibody (Fig. 1) revealed increased tyrosine phosphorylation of p55 Shb upon TCR stimulation in Jurkat-neo cells, observed with equal intensity both when immunoprecipitating native cell extracts and also after boiling the cell extracts in the presence of SDS. The latter procedure decreases the association of proteins to Shb, thus distinguishing between the presence of Shb-associated proteins and Shb itself in immunoprecipitates. Absence of an effect by boiling in SDS indicates that the 55-kDa tyrosine-phosphorylated product is indeed Shb. We thus conclude that Shb becomes tyrosine-phosphorylated upon TCR stimulation.

Association between Shb and p36/38 LAT in CD3-stimulated T Cells—To identify the p36/38 phosphotyrosine protein previously shown to associate with Shb (13) as the recently cloned linker protein LAT (2), we have utilized a double immunopre-
Shb and T Cell Receptor-mediated NFAT Activation

To characterize the interaction between Shb and LAT, peptide displacement experiments were performed, in which the binding of LAT to immobilized Shb PTB domain fusion protein (gstp55ShbΔsh2) was determined. Three peptides encompassing possible tyrosine phosphorylation sites of LAT (Tyr-45, pLAT-1; Tyr-127, pLAT-2; and Tyr-171, pLAT-3) with Asp in position 2 or 3 upstream of the tyrosine (thus conforming with the Shb PTB domain consensus binding sequence) were synthesized and used for experimentation. In Fig. 2C, inhibition of LAT binding to the Shb PTB domain was noted with all three peptides. The pLAT-1 peptide produced a 45% inhibition of binding at 0.2 mM, whereas the other two peptides achieved an inhibition of more than 90% at this concentration. LAT binding to the Shb PTB domain was also completely blocked by free phosphotyrosine. When the corresponding experiment was performed adding the peptides at a final concentration of 50 μM, the pLAT-1 peptide did not inhibit the binding of LAT to the Shb PTB domain fusion protein, whereas the inhibition of association in the presence of the pLAT-2 and pLAT-3 peptides was 60 and 86%, respectively. The data thus raise the possibility of multiple binding sites for the Shb PTB domain on tyrosine-phosphorylated LAT including tyrosines 127 and 171.

Effects of Expression of R522K-Shb in Jurkat Cells on Tyrosine Phosphorylation—To further assess the role of the Shb SH2 domain for TCR signaling, Jurkat cells were transfected with a cDNA construct containing an R522K mutation in the SH2 domain of Shb as described previously (13). Subsequent clonal selection for neomycin resistance yielded 60 clones, several of which showed overexpression of R522K Shb. Nine clones overexpressing R522K Shb were initially assessed for tyrosine phosphorylation of cellular proteins in response to CD3 stimulation, and seven of these exhibited a distinctly decreased response compared with the control Jurkat cells (results not shown). Two of these clones showing an altered response to CD3 stimulation were named Jurkat R522K-2 and R522K-3 and investigated further in the present study. We have previously described the properties of one clone overexpressing an R522K Shb called Jurkat R522K-1 (13). Fig. 3 shows the effect of the R522K mutation in the Shb SH2 domain on the tyrosine phosphorylation of proteins in whole cell lysates after stimulation with anti-CD3 antibodies in the two clones, as determined by Western blot analysis using the phosphotyrosine antibody 4G10. The Jurkat R522K-2 and -3

![Fig. 2. Association of Shb and p36/38 LAT in CD3-stimulated T cells.](image)

![Fig. 3. Tyrosine and MAP kinase phosphorylation of whole cell lysates of Jurkat T cells overexpressing R522K Shb.](image)
cells show weaker tyrosine phosphorylation in response to TCR stimulation by CD3 cross-linking compared with the neomycin resistant control cells of proteins migrating as 160, 70, and 66 kDa and p36/38 LAT. Especially the Jurkat R522K-2 clone displays very poor phosphorylation in response to TCR stimulation, and these cells exhibited a higher degree of R522K Shb expression than the R522K-3 cells (Fig. 3) and the R522K-1 cells (13). These results are in fair agreement with the previously observed response to expression of R522K Shb in the Jurkat R522K-1 cells (13).

**Phosphorylation of LAT Is Dependent on the Shb SH2 Domain**—To further investigate the effects of the Shb R522K mutation on the tyrosine phosphorylation of LAT, anti-CD3-stimulated Jurkat-neo or Jurkat R522K-2 cells were lysed, and proteins were immunoprecipitated with α-LAT antibodies. The blot was subsequently probed with the anti-phosphotyrosine antibody and anti-LAT antibodies (Fig. 4). The data demonstrate that even though the levels of LAT are similar in all lanes, the phosphorylation of LAT in response to CD3 stimulation is abolished in the cells expressing the Shb mutant with a defective SH2 domain. These results suggest that Shb with a functional SH2 domain is of significance for linking LAT to tyrosine kinases in the T cell and for the subsequent phosphorylation of LAT upon TCR stimulation. Association between Shb and LAT might therefore be an important early event in the signaling cascade following TCR engagement in T cells.

To determine the degree of tyrosine phosphorylation of LAT associated with wild-type and mutant Shb, we performed SDS-boil immunoprecipitation experiments on CD3-stimulated/unstimulated Jurkat-neo cells and Jurkat R522K-2 cells, as described previously (Fig. 1). Western blot analyses of these α-Shb immunoprecipitates revealed in the Shb SH2-defective clone, Jurkat R522K-2, no increased phosphorylation of either Shb or LAT after TCR stimulation. In the normal Jurkat-neo clone we saw a marked increase in the phosphorylation of LAT after TCR stimulation, and tyrosine-phosphorylated LAT present in these immunoprecipitates was largely dissociated by SDS treatment, indicating that it is in association with Shb.

Conversely, Jurkat cells overexpressing a mutant LAT (Y171F/Y191F) (2), with two tyrosines mutated to phenylalanine, displayed reduced tyrosine phosphorylation of both p55 and p66 Shb in response to TCR stimulation despite the presence of similar amounts of Shb in the immunoprecipitates (Fig. 5A). Because the transfected wild-type and mutant LAT were tagged with a Myc epitope, the amounts of LAT in the corresponding Shb immunoprecipitates were determined by blotting for Myc (Fig. 5A). Similar amounts of epitope-tagged LAT were present in the immunoprecipitates from both the wild-type and mutant LAT clones, regardless of whether the cells were CD3-stimulated or not. In the cell lysates, the phosphorylation of LAT in absolute and relative amounts was reduced in response to CD3 stimulation in the cells expressing the mutant LAT to a degree similar as that observed in the Shb immunoprecipitates (Fig. 5B). It seems that the association between LAT and Shb is crucial for the phosphorylation of both Shb and LAT in response to TCR stimulation. We therefore suggest that the association between Shb and LAT creates a signaling complex that might also involve other adaptor proteins in the T cell signaling pathway.

**Shb Is Important for the MAP Kinase Signaling Pathway in T Cells**—To assess the effects of the R522K mutation in the Shb SH2 domain on the activation of MAP kinases extracellular signal-regulated protein kinase-1 and -2 upon TCR stimulation, blots from cell extracts of CD3-stimulated and unstimulated Jurkat R522K-2, Jurkat R522K-3, and Jurkat-neo cells were probed with an antibody that recognizes phosphorylated MAPK (Fig. 3). The degree of phosphorylation upon stimulation equals the degree of activation. Total MAPK is also shown as a control of equal loading. Jurkat R522K-2 and -3 cells displayed a decreased phosphorylation of both extracellular signal-regulated protein kinase-1 and -2 MAP kinases (p42 and p44) after CD3 stimulation compared with the Jurkat-neo cells.

It is noteworthy that the altered pattern of tyrosine phosphorylation in cells with a defective Shb SH2 domain diminishes the activation of the MAP kinases in Jurkat T cells.

**Phosphorylation of PLC-γ1 Is Affected by the R522K Mutation in Shb**—Because PLC-γ1 is an important component of TCR-mediated signaling, and because PLC-γ1 is known to be activated by tyrosine phosphorylation in TCR-activated cells, we decided to investigate the potential interactions between Shb and PLC-γ1. PLC-γ1 was present in α-Shb immunoprecipi-$\text{Fig. 4. Phosphorylation of LAT in response to CD3 stimulation in Jurkat cells overexpressing R522K Shb.}$ Jurkat-neo and Jurkat R522K-2 cells (10^7) without treatment (−) or stimulated for 2 min with CD3 cross-linking antibody (+) were lysed with Brij lysis buffer, nuclei were pelleted, and supernatants were subjected to immunoprecipitation using anti-LAT antibody. The immunoprecipitates were resolved by SDS-PAGE, and the blot was first probed with 4G10 anti-phosphotyrosine antibody and then reprobed with anti-LAT antibody. The positions of p36/38 LAT and IgG heavy chain are indicated.

**Fig. 5. Phosphorylation of Shb in response to CD3 stimulation in Jurkat cells overexpressing a mutant form of LAT (Y171F/Y191F).** A, Jurkat cells (10^7) overexpressing wild-type (WT) or mutant (Y171F/Y191F) (YYFF) LAT were unstimulated (−) or stimulated with CD3 antibody (+) for 2 min and lysed in Brij lysis buffer. Supernatants were immunoprecipitated using anti-Shb antibody. Immunoprecipitates were resolved by SDS-PAGE, and the blot was probed with 4G10 phosphotyrosine, anti-Shb, and Myc antibodies. Positions of LAT, p55, and p66 Shb are indicated. B, the cell lysates corresponding to the immunoprecipitates in A were subjected to Western blot analysis for phosphotyrosine (4G10) and LAT. The tyrosine phosphorylation levels and total cellular contents of LAT are shown.
precipitated for Shb, and subjected to Western blot analysis for PLC-

amounts of Jurkat-neo and Jurkat-R522K-2 cells were lysed, immuno-

cipitated using PLC-

lysis buffer, nuclei were pelleted, and cell extracts were immunopre-

cipitates and tyrosine phosphorylation PLC-

g of p55Shb R522K fusion protein (13) and

phosphorylation compared with the neo control cells.

To address the significance of the R522K mutation for the association between Shb and PLC-γ1, the presence of PLC-γ1 in the anti-Shb immunoprecipitates of Jurkat-neo and Jurkat-

R522K-2 cells was determined (Fig. 6C). As noted, the presence of PLC-γ1 was similar in all immunoprecipitates, regardless of CD3 stimulation or whether these were from control or Shb-

mutant cells. Similarly, the binding of PLC-γ1 to immobilized p55 Shb R522K fusion protein was equal regardless of the presence of 20 mM phosphorylase during the binding reaction or whether the cells had been CD3-stimulated or not (Fig. 6D).

Thus, the association between Shb and PLC-γ1 appears not to require a functional Shb SH2 domain, is phosphorylase-independent, and does not require CD3 stimulation. One possible mode of interaction is that between proline-rich sequence of Shb and the SH3 domain of PLC-γ1. A functional Shb SH2 domain is nevertheless required for efficient tyrosine phosphorylation of PLC-γ1.

A Functional Shb SH2 Domain Is Essential for an Increase in Ca2+ in Response to CD3 Stimulation—It is known that PLC-γ1 regulates the hydrolysis of phosphatidylinositol phosphate and thereby generates inositol 1,4,5-tris-phosphate and diacylglycerol, of which the former increases cytoplasmic calcium levels. Therefore Jurkat R522K-2 and Jurkat-neo cells were stimulated with anti-CD3 antibodies, whereas intracellular Ca2+ was measured using a time-sharing dual wavelength fluorometric approach (Fig. 7). After a delay of about 30 s, anti-CD3 induced a pronounced [Ca2+]i response in Jurkat-neo but not in Jurkat R522K-2 cells. These results indicate that the interactions between PLC-γ1 and the TCR involve Shb and are essential for the increase in intracellular Ca2+.

Effects on the Activation of the NFAT Factor and IL-2 Expression by the Shb R522K Mutation—The IL-2 promoter contains several regulatory elements that can bind different transcription factors, such as NFAT, Oct, AP-1, and NF-κB. To investigate the importance of the Shb SH2 domain for T cell receptor signaling downstream of MAP kinases and PLC-γ1, the effects on transcription from the IL-2 regulatory NFAT binding element after TCR stimulation were studied. For this we have used a reporter gene construct composed of a triplet of the NFAT binding site from the IL-2 promoter region coupled to

phosphorylase. As displayed in Fig. 6B, the mutant clone R522K-2 displays a significantly decreased level of PLC-γ1 phosphorylation compared with the neo control cells.

states, but not after immunoprecipitation with preimmune se-

serum as assessed by Western blot analysis using an antibody reac-

tive with PLC-γ1 regardless of whether the cells were CD3-stimulated or not (Fig. 6A). To see whether the phosphory-

lation and activation of PLC-γ1 is negatively affected by a deffective Shb SH2 domain, we performed immunoprecipitation experiments using a PLC-γ1 antiserum on the Jurkat R522K-2 and Jurkat-neo cells followed by Western blot analysis for

FIG. 6. Co-immunoprecipitation of PLC-γ1 in anti-Shb immu-

noprecipitates and tyrosine phosphorylation PLC-γ1 in Jurkat

cells overexpressing R522K Shb. A. Jurkat cells (10⁷) without treat-

ment (−) or stimulated for 2 min with CD3 cross-linking antibody (+) were lysed with Triton lysis buffer, nuclei were pelleted, and superna-

tants subjected to immunoprecipitation using either normal rabbit se-

rum (NRS) or anti-Shb antibody (Shb). The immunoprecipitates were resolved by SDS-PAGE, and the blot was probed with anti-PLC-γ1/Nck antibody. B. Jurkat-neo and Jurkat R522K-2 cells (10⁵) unstimulated (−) or incubated with CD3 antibody for 2 min (+) were lysed in Triton lysis buffer, nuclei were pelleted, and cell extracts were immunopre-

cipitated using PLC-γ1 antibody. Immunoprecipitates were resolved by SDS-PAGE, and the blot was subsequently probed with 4G10 anti-

phosphotyrosine antibody and PLC-γ1 antibody as indicated. C, equal amounts of Jurkat-neo and Jurkat-R522K-2 cells were lysed, immuno-

precipitated for Shb, and subjected to Western blot analysis for PLC-γ1. D, cell extracts from nonstimulated (−) and CD3-stimulated (+) Jurkat cells were incubated in the absence or presence of 20 mM phosphorylase (pH 7.5) with 5 μg of p55Shb R522K fusion protein (13) and subsequently analyzed for the presence of PLC-γ1 by Western blot analysis.

FIG. 7. Concentration of cytoplasmic [Ca2+]i, after CD3 stimu-

lation in Jurkat-neo and Jurkat-R522K-2 cells. Fura-2-loaded Jurkat-neo and Jurkat R522K-2 cells (2.5 × 10⁶ cells/1 ml of medium) were stimulated with CD3 antibody (9.2 μg/ml) as indicated. Means ± S.E. for three experiments are shown.
a CAT reporter, because this regulatory element is particularly important for T cell receptor signaling (17). Jurkat cells were transiently transfected with vector alone (pcDNA1) or mutant R522K Shb inserted into this vector, together with the NFAT-CAT construct. All values were normalized relative TPA + CD3-stimulated cells transfected with pcDNA1 only after subtraction of the corresponding values of nonstimulated cells. Fig. 8A demonstrates that the cells expressing a mutation in the SH2 domain of Shb display no activation of this element upon TCR stimulation, whereas in the control cells, a 3-fold increase in the NFAT-mediated transcription can be seen after TCR stimulation compared with TPA treatment alone. CD3 stimulation without concomitant TPA treatment resulted in a weak stimulation of NFAT activity (results not shown).

When the Jurkat-neo, Jurkat R522K-2 and -3 clones were analyzed for NFAT activation in a manner similar to that described above, it was observed that both Jurkat R522K-2 and -3 cells display a poor NFAT-mediated induction of transcription.
tion in response to CD3 cross-linking compared with the Jurkat-neo cells (Fig. 8D). The NFAT activity in these experiments was normalized in a different manner than in Fig. 8A, because the different clones displayed a large variation in their transfection efficiency, thus requiring an intraclonal normalization procedure.

We have also looked at the endogenous IL-2 levels in Jurkat cells transiently transfected with vector, wild-type Shb, or mutant R522K Shb, using a phycoerythrin-conjugated IL-2 antibody and FACS analysis. The cells were stimulated for 6 h with CD3+TPA or with TPA + ionomycin or left unstimulated. The cells were then fixed, permeabilized together with PE-oIL-2 antibody, and analyzed by FACS. We observed a 4-fold increase in IL-2 expression in the vector and wild-type Shb-transfected cells after stimulation with CD3 and TPA, whereas there was no increase in IL-2 production in the R522K Shb transfected cells after stimulation with CD3+TPA (Fig. 8C). To assess the maximal IL-2 response, we stimulated cells with ionomycin+TPA, which produced a 18–24-fold increase of the IL-2 contents in the R522K Shb, wild-type Shb, and control transfected cells (Fig. 8C).

The results in Fig. 8 indicate that the adaptor protein Shb plays an important role in the signaling cascade that leads to activation of transcription via the NFAT element of the IL-2 gene following TCR stimulation.

**DISCUSSION**

We have previously established a role for Shb in TCR signaling by describing SH2 domain-dependent binding of Shb to the TCR ζ-chain, PTB domain-dependent association of Shb to p36/38 LAT, and proline-rich/SH3 domain interactions with Grb2 (13, 14). In this report, we describe Shb signaling, through MAPK and Ca2+, eventually causing activation of the NFAT element in the IL-2 gene promoter.

To elucidate the importance of the previously observed interaction between the Shb SH2 domain and the ζ-chain of the TCR (13) we have introduced a point mutation in the SH2 domain of Shb and expressed this mutant. As a consequence, a functional SH2 domain. The results indicate that Shb and p36/38 LAT form a complex that is of importance for the phosphorylation of both Shb and LAT upon TCR engagement and further signaling via both the Ras and Ca2+ pathways. p36/38 LAT is a membrane-associated protein that has been shown to bind Grb2, Sos (possibly via Grb2), PLC-γ1, and the p85 subunit of phosphoinositide 3-kinase (12, 24). It is also one of the most prominently tyrosine-phosphorylated proteins detected following TCR engagement (25). It was previously thought that this p36/38 protein might be responsible for linking PLC-γ1 and the Grb2-Sos complex to the TCR. However, cloning of the p36/38 linker revealed a protein with several tyrosine phosphorylation sites but no SH2 or other binding domains. We currently propose that Shb might be an adaptor participating in linking p36/38 LAT with the TCR ζ-chain via the SH2 and PTB domains of Shb. The Shb-LAT complex after TCR stimulation appears capable of serving as a substrate for protein tyrosine kinases, such as ZAP70, that previously has been shown to phosphorylate LAT (2). The kinase can then mediate the phosphorylation of the Shb-LAT complex, and other signaling proteins associated with it, such as PLC-γ1.

Our results do not exclude the involvement of other pathways of significance for interactions between LAT and ZAP70 or the TCR ζ-chain resulting in phosphorylation of LAT. The Jurkat R522K-3 clone exhibiting moderately elevated contents of R522K Shb demonstrated diminished but detectable tyrosine phosphorylation of LAT in response to TCR activation. Such a finding raises the possibility of other, Shb-independent mechanisms for targeting LAT to appropriate sites adjacent to the TCR. Besides the lipophilic properties of LAT itself, the involvement of the 3BP2 adaptor molecule could be of relevance in this context (26).

The phosphotyrosine-dependent association between Shb and LAT observed previously (13) and in the present study and the peptide displacement data demonstrated in the present study using the pLAT-1, -2, and -3 peptides suggest that Shb associates with one or several of the many potential tyrosine phosphorylation sites present in LAT (2). Although the Tyr-171 peptide efficiently inhibited the binding of LAT to the Shb PTB domain, this position cannot alone mediate this interaction, because binding of LAT to Shb was detected in the cells expressing the Y171F/Y191F mutant, albeit at a reduced level, suggesting a modest decrease of LAT-Shb association with this mutant. Another potential site of interaction between these two molecules is tyrosine 127. Association between Shb and LAT was noted in nonstimulated cells, suggesting that basal phosphorylation of LAT without CD3 stimulation is sufficient for at least partial Shb association.

The data show that Shb and PLC-γ1 associate in T cells and that this interaction is independent of TCR stimulation. A possible mode of interaction is that between the proline-rich sequences in the ζ terminus of Shb and the SH3 domain of PLC-γ1. However, expression of a mutant LAT also diminishes its association with PLC-γ1 (2), implying a functional relationship between those two molecules as well. In order to reconcile these observations, we propose a model in which a trimeric complex between Shb, LAT, and PLC-γ1 exists. One potential mechanism for targeting such a complex to the TCR ζ-chain is via the SH2 domain of Shb, and thus the complex will be brought in contact with TCR-associated tyrosine kinases that activate PLC-γ1 by phosphorylation. Active PLC-γ1 is known to regulate intracellular Ca2+ through inositol phospholipids, and as anticipated, the Jurkat R522K-2 cells are unable to increase the Ca2+ concentration in response to TCR activation.

Shb has previously been shown to associate with Grb2 (13), and because Grb2 efficiently binds via its SH2 domain tyrosine-phosphorylated LAT, it is conceivable that a trimeric protein complex also exists between Shb, LAT, and Grb2. Again, this complex may at least partly be targeted to the TCR ζ-chain by the Shb SH2 domain, Sos, a guanine nucleotide exchange factor for Ras, also associates with Grb2 and LAT (2), thus connecting signaling through Shb with the Ras/MAPK pathway. This is further supported by the significantly decreased degree of MAP kinase activation in response to TCR stimulation in the Jurkat R522K mutant clones.

It is known that the Ras/MAPK-pathway and the Ca2+ pathway cooperate in T cells to activate the IL-2 promoter through the transcription factors NFAT and AP-1. The Ras/MAPK pathway can activate AP-1 and also synergize with the calcium pathway to activate the T cell-specific transcription factor NFAT. NFAT proteins translocate from the cytosol to the nucleus in TCR-activated cells and can combine with AP-1 to form a transcription factor complex (27) and initiate IL-2 gene transcription. There have been reports on a number of signaling proteins affecting NFAT activation: for example, p36/38 LAT (2), p21 Ras (28), Vav, and SLP-76 (3). Shb has been shown to play a role in both the Ras/MAPK pathway and the calcium signaling pathway, and it was not surprising that TCR-mediated activation of NFAT is also abolished in Jurkat cells expressing an R522K mutation in the Shb SH2 domain. Both stable and transient transfectants failed to activate NFAT in response to TCR engagement.

The results presented in this study provide evidence that
Shb is an adaptor protein involved early in T cell receptor signaling pathways through its interactions with the T cell receptor ζ-chain, LAT, Grb2, and PLC-γ1. Other proteins of importance for T cell signaling are SLP-76, Vav, Cbl, and CrkL (3, 29) and the tyrosine kinases Lck, Fyn, and ZAP70 (1). At present, possible interactions between these proteins and Shb have not been elucidated. The participation of Shb in other signaling pathways than MAP kinases and Ca^{2+} in Jurkat cells are potentially of great interest and will be addressed in future studies.

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