Positive Regulation of T Cell Activation and Integrin Adhesion by the Adapter Fyb/Slap

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The molecular adapter Fyb/Slap regulates signaling downstream of the T cell receptor (TCR), but whether it plays a positive or negative role is controversial. We demonstrate that Fyb/Slap-deficient T cells exhibit defective proliferation and cytokine production in response to TCR stimulation. Fyb/Slap is also required in vivo for T cell–dependent immune responses. Functionally, Fyb/Slap has no apparent role in the activation of known TCR signaling pathways, F-actin polymerization, or TCR clustering. Rather, Fyb/Slap regulates TCR-induced integrin clustering and adhesion. Thus, Fyb/Slap is the first molecular adapter to be identified that couples TCR stimulation to the avidity modulation of integrins governing T cell adhesion.

Engagement of the TCR by antigen initiates intracellular signaling cascades that result in T cell activation, differentiation, acquisition of effector function, or apoptosis (*1*). Dramatic changes to the cytoskeleton and transcription are the outcomes of these cascades, but the molecular mechanisms that link TCR stimulation to downstream pathways are not well understood. Signals from cell surface receptors are coupled to distal signaling pathways by adapter proteins, molecules that contain multiple protein-protein interaction domains. Several adapter molecules have been identified that have positive effects on TCR signaling, including Grb2, LAT, GADS, and Slp-76 (*2*, *3*). Others, such as c-Cbl and Cbl-b, have a negative regulatory function.

Fyb/Slap is an adapter protein expressed in T cells and myeloid cells that contains a proline-rich region, tyrosine phosphorylation sites, an EVH1-binding domain, and an SH3-like domain (*4–6*). It is expressed as two isoforms of 120 kD and 130 kD, which differ in expression pattern between thymocytes and mature T cells (*7*). Fyb/Slap binds to the Src family kinase Fyn, the adapter protein Slp-76, which is required for thymocyte development, TCR-dependent mitogen-activated protein kinase (MAPK) activation and calcium flux (*8–10*), and Ena/VASP proteins (*6*), which are modulators of the cytoskeleton. The functional role of Fyb/Slap has remained controversial because overexpression studies have shown Fyb/Slap to be either a positive regulator (*4*, *7*, *11*) or a negative regulator (*5*, *12*) of interleukin-2 (IL-2) production, depending on the experimental system used.

To elucidate Fyb/Slap's biological role in T cell function, we used *Rag2*$^{-/-}$ blastocyst complementation to generate gene-targeted chimeric mice lacking Fyb/Slap expression in mature T and B lymphocytes (*13*). *Fyb/slap*$^{-/-}$ chimeras displayed normal numbers and differentiation of thymocytes and B cells; however, the relative numbers of CD4$^+$ T cells in the lymph nodes and CD4$^+$ and CD8$^+$ T cells in the spleen were reduced in *fyb/slap*$^{-/-}$ chimeras as compared to *fyb/slap*$^{+/-}$ chimeric controls (*13*). These results indicate that lymphocyte development is normal in the absence of Fyb/Slap but that this adapter may be required for peripheral T cell homeostasis in vivo.

To determine whether Fyb/Slap positively or negatively influences lymphocyte function, we measured the proliferative responses of purified lymphocytes from *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ chimeric mice in vitro (*14*). Consistent with the lack of expression of Fyb/Slap in B cells (*4*), B cell proliferation was comparable between Fyb/Slap-deficient and control animals. Purified T cells from *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ chimeras responded equally to phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (Fig. 1, A and B). However, stimulation with monoclonal antibody (mAb) to CD3ε (anti-CD3ε) alone, or anti-CD3ε plus antibody to CD28 (anti-CD28) mAbs, revealed a dramatic decrease in the proliferation of *fyb/slap*$^{-/-}$ T cells compared to *fyb/slap*$^{+/-}$ T cells at both 24 hour and 48 hour time points (Fig. 1, A and B). Using the same stimulation conditions, IL-2 (Fig. 1C) and interferon-γ (IFN-γ) (Fig. 1D) production were also strongly reduced in *fyb/slap*$^{-/-}$ T cells. In addition, the up-regulation of CD25 (IL-2 receptor α chain) and CD69 (Fig. 2E) was defective in Fyb/Slap-deficient T cells. Consistent with the impaired expression of the high affinity IL-2R, addition of exogenous IL-2 only partially rescued the proliferative defect in *fyb/slap*$^{-/-}$ T cells. These data demonstrate that Fyb/Slap is an important positive regulator of T cell activation and proliferation and of cytokine production.

To investigate whether the requirement for Fyb/Slap in T cell activation and proliferation in vitro translated into a defective immune response in vivo, we immunized the chimeric mice with the T cell–dependent (TD) antigen 4-hydroxy-3-nitrophenylacetyl–ovalbumin (NP-OVA) (*15*). Whereas *fyb/slap*$^{+/-}$ chimeras produced high titers of NP-specific immunoglobulin G1 (IgG1) Ab, the IgG1 response was completely absent in *fyb/slap*$^{-/-}$ chimeras (Fig. 1F). Moreover, in mice infected with vesicular stomatitis virus (VSV), virus-specific IgG titers showed a gene-dosage dependent decrease in *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ chimeras as compared to *fyb/slap*$^{+/+}$ controls (*15*). The block in antibody production was due to a defect in T cells and not B cells, because *fyb/slap*$^{-/-}$, *fyb/slap*$^{+/-}$, and *fyb/slap*$^{+/+}$ mice produced comparable 2,4,6-trinitrophenyl (TNP)–specific IgG3 after immunization with the T cell–independent (TI) polyvalent antigen TNP-Ficoll (Fig. 1G). We conclude that Fyb/Slap is essential for the delivery of effective T cell help and for the generation of functional immune responses to TD antigens in vivo.

To identify the molecular mechanism that accounts for the functional defects in *fyb/slap*$^{-/-}$ T cells, we analyzed signaling pathways downstream of TCR stimulation. *Fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells exhibited comparable calcium fluxes after anti-CD3ε crosslinking (Fig. 2A) (*14*). Moreover, stimulation of *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ thymocytes or peripheral T cells with anti-CD3ε with or without anti-CD28 revealed no apparent differences in total tyrosine phosphorylation or in expression levels or tyrosine phosphorylation of LAT, PLCγ1, Slp-76, c-Cbl, Vav1, Fyn, or Lck (*14*). Both Fyn and Lck kinase activities were normal. Lastly, the phosphorylation kinetics and activities of Erk1/Erk2, SAPK/JNK, and p38 were also comparable between *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$

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peripheral T cells (Fig. 2B) (*14*). Thus, despite the requirement of Fyb/Slap for T cell activation and proliferation, the major signaling pathways downstream of the TCR appear intact in the absence of Fyb/Slap.

Overexpression studies have suggested a role for Fyb/Slap in cytoskeletal rearrangement downstream of the TCR (*6*). In addition, Fyb/Slap co-localizes with Ena/VASP proteins that have been shown to control cytoskeletal reorganization, cell motility, and cell adhesion (*6*, *16*–*18*). Clustering of the TCR/CD3 chains after T cell stimulation is required for efficient T cell activation and is dependent on the actin cytoskeleton (*19*). To further explore the molecular mechanism by which Fyb/Slap regulates T cell activation and immune responses, we investigated whether Fyb/Slap was involved in TCR-induced CD3 clustering or actin polymerization (*14*). The extent of TCR-mediated antigen receptor clustering was comparable between *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells after anti-CD3ε stimulation (Fig. 2, C and D). TCR-induced F-actin clustering was also comparable between *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells (Fig. 2, C and D). Thus, Fyb/Slap is not required for TCR-triggered antigen receptor clustering and actin polymerization.

In addition to clustering of the TCR itself, a second cytoskeleton-dependent process triggered by the TCR is the clustering of integrin receptors leading to T cell adhesion (*20*). Adhesion of T cells via integrin receptors is critical for T cell development, migration, and activation, and integrins such as LFA-1 have been demonstrated to be important for in vitro T cell proliferation (*21*, *22*). In resting T cells, integrins are maintained in a relatively inactive state. TCR ligation initiates a poorly understood process of "inside-out" signaling that induces ligand binding (*23*, *24*). Inside-out signaling and integrin binding are required to stabilize the interaction between T cells and antigen-presenting cells and to form the immune synapse (*25*). TCR-induced clustering of the β2 integrin LFA-1 in the cell membrane increases integrin avidity for its ligand, intercellular adhesion molecule−1 (ICAM-1), and is an important mechanism for the induction of lymphocyte adhesion (*20*). To examine whether Fyb/Slap plays a role in integrin clustering, we assessed LFA-1 distribution on the cell surface after CD3ε stimulation (*14*). The proportion of *fyb/slap*$^{-/-}$ T cells that exhibited LFA-1 clusters after stimulation was markedly reduced compared to *fyb/slap*$^{+/-}$ T cells (Fig. 2, C and D). The clustering defect in Fyb/Slap-deficient cells is not due to a change in clustering kinetics, because *fyb/slap*$^{-/-}$ T cells also exhibit defective clustering at 15 min or 45 min, whereas maximal clustering of *fyb/slap*$^{+/-}$ T cells is seen at 30 min. Thus, Fyb/Slap plays a specific role in coupling TCR stimulation to LFA-1 lateral mobility in the membrane.

To examine whether the defect in LFA-1 clustering seen in *fyb/slap*$^{-/-}$ T cells has functional relevance for integrin-mediated adhesion, we stimulated *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells with anti-CD3ε and assayed adhesion to plates coated with recombinant mouse ICAM-1 (mICAM-1) and human ICAM-2 (hICAM-2) (*26*). Adhesion to hICAM-2 was performed using a parallel plate flow chamber that produces a reproducible force on adherent cells and quantifies the strength of cell adhesion. *Fyb/slap*$^{-/-}$ T cells stimulated with anti-CD3ε were defective in adhering to mICAM-1 and hICAM-2 (Fig. 3, A and B). TCR-induced adhesion mediated by other integrins was also defective in *fyb/slap*$^{-/-}$ T cells. This was the case with adhesion to fibronectin, mediated by several integrins including α5β1 and αvβ3, and adhesion to mouse vascular cell adhesion molecule–1 (mVCAM-1), mediated by α4 integrins (Fig. 3, C and D). The cell surface expression of LFA-1 (CD11a), CD18, CD11c, α4 integrin, LFA-2, ICAM-1, L-selectin, platelet endothelial cell adhesion molecule–1 (PECAM-1), and CD30 was not reduced in *fyb/slap*$^{-/-}$ T cells compared to *fyb/slap*$^{+/-}$ controls. In all cases, treatment with PMA, magnesium, or manganese induced comparable adhesion to controls, indicating that the defect in TCR-induced adhesion is due to a block in inside-out signaling between the TCR and integrins and is not due to an intrinsic defect of the integrins (Fig. 3, A through D). Thus, in the absence of Fyb/Slap, communication between the antigen receptor and multiple integrins is impaired, inhibiting the coupling of TCR signaling to T cell adhesion.

In conclusion, we have shown both in vitro and in vivo that Fyb/Slap is a critical positive regulator of T cell activation and function. *Fyb/slap*$^{-/-}$ T cells are defective in TCR-mediated activation, proliferation, and cytokine production. In vivo, *fyb/slap*$^{-/-}$ chimeric mice display impaired immunity to TD antigens. Functionally, Fyb/Slap has no apparent role in the activation of known TCR signaling pathways, TCR clustering, or F-actin formation. Rather Fyb/Slap controls communication between the TCR and integrins and regulates integrin clustering and integrin-mediated adhesion in response to TCR stimulation. Thus, Fyb/Slap is the first molecular adapter to be identified that couples TCR signaling to integrin activation, inside-out

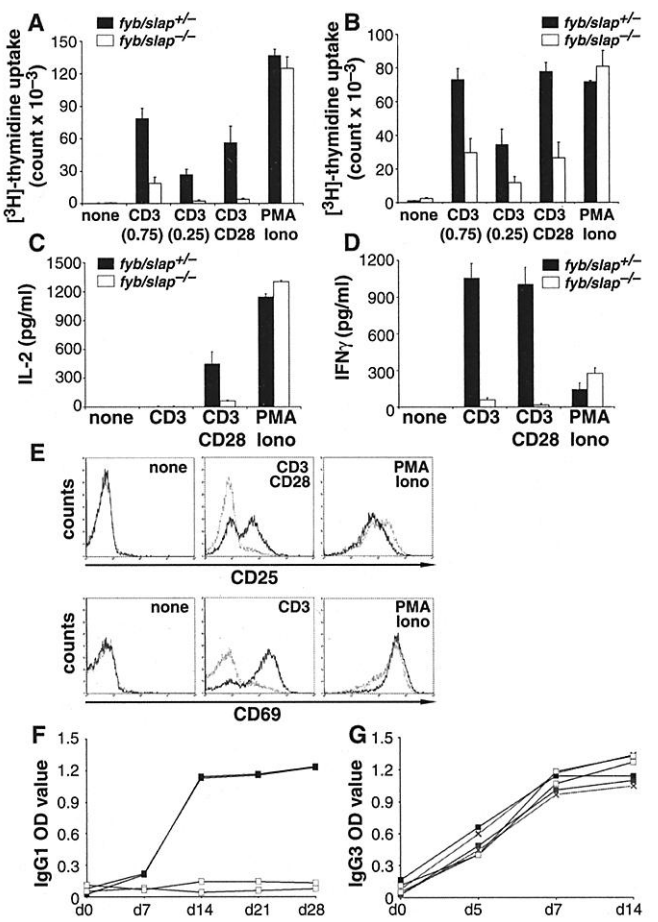

Fig. 1. Fyb/Slap is a critical regulator of T cell activation. (**A** and **B**) Proliferation of purified lymph node T cells. Cells were stimulated with plate-bound anti-CD3ε (0.75 μg/ml or 0.25 μg/ml), anti-CD3ε (0.25 μg/ml) and soluble anti-CD28 (1 μg/ml), or with PMA (50 ng/ml) plus ionomycin (100 ng/ml). Proliferation was determined by [^{3}H]thymidine incorporation after 24 hours (A) or 48 hours (B). (**C** and **D**) Cytokine production. T cells were stimulated as in (A) for 48 hours (C) or 24 hours (D). Levels of IL-2 (C) and IFN-γ (D) production were determined by ELISA. (**E**) Activation marker up-regulation. Purified *fyb/slap*$^{+/-}$ (solid line) and *fyb/slap*$^{-/-}$ (dotted line) T cells were stimulated as indicated for 48 hours (CD25, top) or 24 hours (CD69, bottom) and analyzed by flow cytometry. (**F**) Impaired response to TD antigen. *Fyb/slap*$^{+/-}$ (solid squares) and *fyb/slap*$^{-/-}$ (open squares) chimeric mice (two mice per genotype) were immunized with NP-OVA intraperitoneally (ip), and serum anti-NP IgG1 titers were determined by ELISA. Each curve represents one individual mouse. One result representative of two independent experiments is shown. (**G**) Normal response to TI antigen. *Fyb/slap*$^{+/+}$ (X), *fyb/slap*$^{+/-}$ (solid squares) and *fyb/slap*$^{-/-}$ (open squares) chimeric mice (two mice per genotype) were immunized with TNP-Ficoll ip, and IgG3 titers were determined by ELISA. Each curve represents one individual mouse.

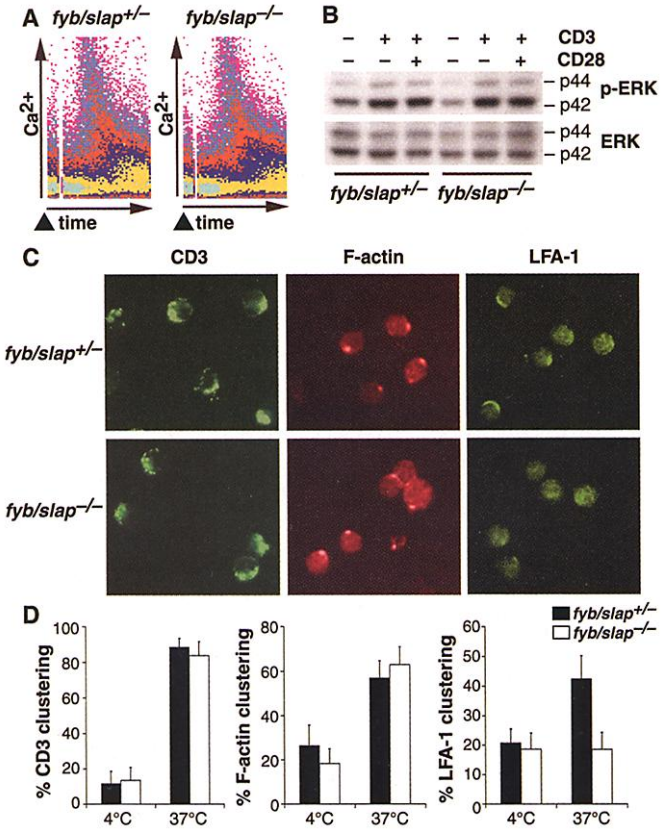

Fig. 2. Normal TCR proximal signaling but impaired integrin clustering in the absence of Fyb/Slap. (**A**) Calcium mobilization. Freshly isolated INDO-1 loaded peripheral T cells from *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ mice were stimulated with anti-CD3ε. Ca^{2+} flux was measured by flow cytometry. The *x* axis shows real time Ca^{2+} release followed for 180 s, and the *y* axis shows the intracellular Ca^{2+} concentration. Arrowheads indicate the time of addition of cross-linking antibody. (**B**) Activation of Erk1 and Erk2. Purified *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells were activated with hamster anti-CD3ε alone or in conjunction with hamster anti-CD28 for 2, 5, (shown) or 10 min. Active Erk1 and Erk2 were detected using a phosphospecific Ab (p-ERK). Levels of total Erk1 and Erk2 protein are shown in the lower panel. Normal Erk activation was confirmed using in vitro kinase assays. (**C**) TCR-induced CD3 clustering, actin polymerization, and LFA-1 clustering. T lymphocytes were incubated with anti-CD3ε and then with biotinylated cross linker at 4°C (control) or 37°C. Cells were cytospun onto slides and fixed with 3.7% PFA. TCR-CD3 clustering was visualized using fluorescein isothiocyanate (FITC)–labeled streptavidin and fluorescence microscopy. F-actin was visualized with tetramethyl rhodamine isothiocyanate (TRITC)–phalloidin, and LFA-1 with a FITC Ab to LFA-1. Representative images after activation for 30 min at 37°C are shown. (**D**) TCR-induced CD3 clustering, F-actin polymerization, and LFA-1 clustering. Cells were stimulated as in (C). Clustering was quantified using a fluorescent microscope (10 fields counted per treatment). Cells were considered to have clustered receptors if the staining pattern showed receptors polarized to one side of the cell.

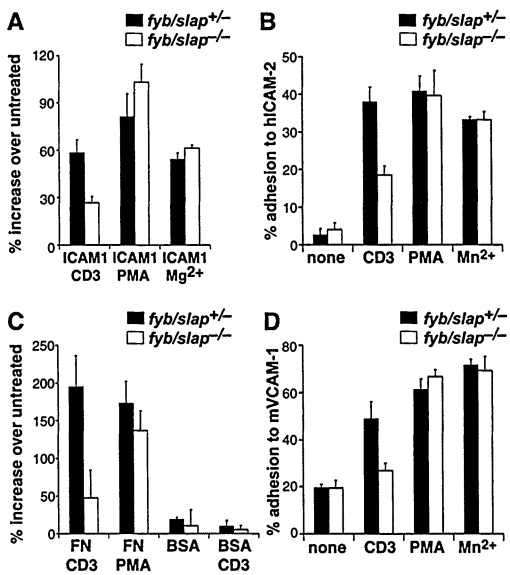

Fig. 3. Fyb/Slap is required for TCR-induced integrin-mediated adhesion. (**A**) T cell adhesion to mICAM-1. Purified *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells were cultured on plates coated with mICAM-1. Adherent cells were counted by trypan blue exclusion. Adhesion of untreated samples to mICAM-1 was 10% of cells plated for both control and *fyb/slap*$^{-/-}$ cells, indicating that loss of Fyb/Slap did not affect baseline adhesion. (**B**) Adhesion of T cells to hICAM-2 under shear stress. Purified *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells were treated as indicated and allowed to settle on hICAM-2 in a parallel plate flow chamber for 10 min at 37°C. Shear stress was then applied and the percentage of cells remaining attached after each shear stress interval determined. For both (B) and (D), adhesion at 10 dynes/cm^2 of shear stress is shown, and comparable results were obtained at 4 dynes/cm^2 of shear force. (**C**) T cell adhesion to fibronectin. Purified *fyb/slap*$^{+/-}$ and *fyb/slap*$^{-/-}$ T cells were cultured on plates coated with fibronectin or BSA (control) and adhesion assays carried out as in (A). Adhesion of untreated cells to fibronectin was 10% of cells plated for both groups. (**D**) Adhesion of T cells to mVCAM-1 under shear stress. Adhesion assays were performed as described in (B).

signaling, and T cell adhesion. These data also demonstrate that integrin clustering can be genetically uncoupled from actin reorganization and define a previously unknown Fyb/Slap-regulated signaling pathway that specifically links TCR stimulation to changes in integrin avidity. On the basis of these studies and other work implicating Fyb/Slap in vasoactive mediator release (*27*), we propose that Fyb/Slap be redesignated Adhesion and Degranulation promoting Adapter Protein (ADAP).

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14. Lymph node T cells were purified using magnetic beads (Dynal, Oslo, Norway) and analyses of proliferation, cytokine production, calcium flux, biochemistry, and clustering were performed as described (*28*).
15. Immunizations and measurements of antibody production were performed as described (*29*). Enzyme-linked immunosorbent assay (ELISA) analysis showed that basal levels of IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE were similar in *fyb/slap*$^{-/-}$ and *fyb/slap*$^{+/-}$ chimeric mice.
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26. T cells in 2.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) were added to plates coated with fibronectin, BSA, or recombinant mICAM-1 (gift of J. Collard, The Netherlands Cancer Institute) and allowed to settle with treatment [medium alone, anti-CD3 (2 μg/ml), or PMA (100 ng/ml)] for 1 hour at 4°C. Samples were incubated at 37°C for 12 min followed by washing with 2.5% BSA in PBS. Adherent cells were released with 10 mM EDTA in calcium-and-magnesium-free PBS and were counted by trypan blue exclusion. Studies addressing adhesion under shear stress were performed as described (*30*).
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Coupling of the TCR to Integrin Activation by SLAP-130/Fyb

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SLAP-130/Fyb (SLP-76–associated phosphoprotein or Fyn-binding protein; also known as Fyb/Slap) is a hematopoietic-specific adapter, which associates with and modulates function of SH2-containing leukocyte phosphoprotein of 76 kilodaltons (SLP-76). T cells from mice lacking SLAP-130/Fyb show markedly impaired proliferation following CD3 engagement. In addition, the T cell receptor (TCR) in SLAP-130/Fyb mutant cells fails to enhance integrin-dependent adhesion. Although TCR-induced actin polymerization is normal, TCR-stimulated clustering of the integrin LFA-1 is defective in SLAP-130/Fyb–deficient cells. These data indicate that SLAP-130/Fyb is important for coupling TCR-mediated actin cytoskeletal rearrangement with activation of integrin function, and for T cells to respond fully to activating signals.

Following the engagement of lymphocyte surface receptors, adapter proteins play critical roles in regulating second messenger signaling cascades (*1*). T cell receptor (TCR) signaling (*2*) and murine thymocyte development (*3, 4*) require the presence of the hematopoietic adapter SLP-76 (SH2 domain containing Leukocyte Phosphoprotein of 76 kD). SLP-76 likely functions as a scaffold for multimolecular complexes that coordinate signaling. However, it is not fully understood how SLP-76 couples TCR-stimulated protein tyrosine kinase (PTK) activity with downstream signals. SLP-76 associates in a TCR-inducible fashion with SLAP-130/Fyb, another hematopoietic-specific adapter (*5, 6*). Although SLAP-130/Fyb has been implicated in T cell migration and rearrangement of the actin cytoskeleton (*7, 8*), overexpression studies seeking to define a functional role for SLAP-130/Fyb in TCR signaling have been inconclusive (*5, 6*).

To address the role of SLAP-130/Fyb in TCR signaling, we generated SLAP-130/Fyb–deficient mice (*9–12*). SLAP-130/Fyb mutant animals are born at expected Mendelian frequencies; the mice are viable, fertile, and show normal growth. Hematopoietic cellularity is normal with the exception of modest thrombocytopenia, a 50% reduction in splenic T cells, and mildly decreased thymocyte number (*13*). We found no differences between wild-type and SLAP-130/Fyb–deficient mice in subsets of thymocytes expressing developmental markers or coreceptors (*13*). Thus, SLAP-130/Fyb, unlike SLP-76, appears dispensable for orderly progression of T cell development.

Because a SLP-76–deficient T cell line exhibits uncoupling of TCR-stimulated protein tyrosine kinases (PTKs) from signals critical for cellular activation (*2*), we examined TCR-stimulated biochemical events in purified SLAP-130/Fyb$^{-/-}$ lymph node and splenic T cells. The earliest detectable biochemical event following engagement of the TCR is activation of members of several families of PTKs. SLAP-130/Fyb$^{-/-}$ T cells exhibit TCR-induced phosphorylation of SLP-76 and PLCγ-1 (Fig. 1A) similar to control cells. Downstream of PTK activation, MAPK up-regulation and calcium elevation are required elements in TCR-dependent cellular activation. Unlike SLP-76–deficient Jurkat cells, SLAP-130/Fyb$^{-/-}$-purified T cells display TCR-dependent MAPK phosphorylation with kinetics comparable to controls (Fig. 1B). We found no decrease in TCR-mediated calcium elevation either by ratiometric flow cytometric assay (Fig. 1C) or by microscopic digital imaging (*14*) of individual T cell fura-2 fluorescence (*15*). From these data, we conclude that SLAP-130/Fyb does not regulate TCR signaling either at the level of PTK activation or in the coupling of PTK activity with key cytoplasmic biochemical events.

TCR signaling results in transactivation of several gene products, including the surface antigens CD25 and CD69. We stimulated purified SLAP-130/Fyb$^{-/-}$ T cells with anti-CD3 for 18 hours, and assessed expression of both markers. Up-regulation of CD25, and to a lesser extent, of CD69, was markedly reduced in SLAP-130/Fyb$^{-/-}$ T cells com-

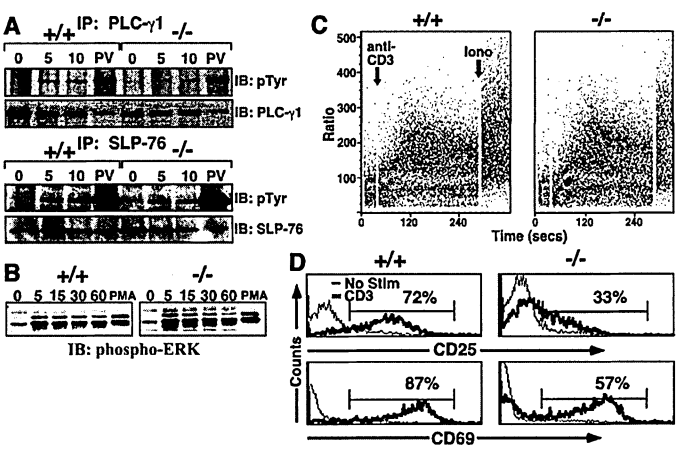

Fig. 1. Proximal signaling occurs in the absence of SLAP-130/Fyb, but early activation antigen expression is impaired. (**A**) Purified splenic T cells (*9–12*) were left unstimulated, or treated with soluble anti-CD3ε (500A2, 5 μg/ml, Pharmingen) for indicated minutes, or with pervanadate (PV) (1 mM $NaVO_4$, 1% H_2O_2) for 2 min. Cellular lysates were prepared in 1% NP-40, and were subjected to immunoprecipitation with anti-SLP-76 or anti-PLCγ1 (Upstate Biotechnology), followed by Western blotting with indicated antisera. In (**B**), cells were left unstimulated or treated with anti-CD3ε (500A2, 5 μg/ml) for the indicated times. Resolved cell lysates were interrogated with anti-phospho-ERK (New England Biolabs). For (**C**), purified lymph node T cells (*9*) were loaded with Indo-1 and stained with anti-CD3ε at 30°C, followed by stimulation with goat anti-hamster (10 μg/ml). Intracellular calcium elevations after TCR stimulation ("anti-CD3") or ionomycin ("Iono") were detected by ultraviolet laser flow cytometry. (**D**) Purified splenic T cells were cultured with platebound anti-CD3ε (2C11, 5 μg/ml) for 18 hours, stained with anti-CD69 or anti-CD25 (Pharmingen), and analyzed by FACS.

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