FYN-T-FYB-SLP-76 Interactions Define a T-cell Receptor ζ/CD3-mediated Tyrosine Phosphorylation Pathway That Up-regulates Interleukin 2 Transcription in T-cells*

(Received for publication, January 28, 1999, and in revised form, April 27, 1999)

Monika Raab‡§, Hyun Kang‡§, Antonio da Silva‡§, Xiaochun Zhu‡§, and Christopher E. Rudd‡§**

From the ‡Division of Tumor Immunology, Department of Cancer Immunology and AIDS, Dana Farber Cancer Institute and Departments of §Medicine and ¶Pathology, Harvard Medical School, Boston, Massachusetts 02115

Protein-tyrosine kinases p56Lck, SYK, and ZAP-70 and downstream adaptors LAT and SLP-76 have been implicated as essential components in T-cell activation. Another lymphoid-specific adaptor FYB/SLAP has also been identified as a predominant binding partner of SLP-76 and the Src kinase FYN-T, although its role in the activation process has been unclear. In this study, we demonstrate that FYN-T selectively phosphorylates FYB providing a template for the recruitment of FYN-T and SLP-76 SH2 domain binding. This interaction is unusual in its distinct cytoplasmic localization and its long term stable kinetics of phosphorylation. Furthermore, we demonstrate for the first time that the co-expression of all three components of the FYN-T-FYB-SLP-76 matrix can synergistically up-regulate T-cell receptor-driven interleukin 2 transcription activity. These findings document the existence of a T-cell receptor-regulated FYN-T-FYB pathway that interfaces with the adaptor SLP-76 and up-regulates lymphokine production in T-cells.

Ligation of CD4/CD8-p56Lck and the T-cell receptor complex (TcRζ/CD3) activates Src protein-tyrosine kinases p56Lck and p59Fyn-T (1) leading to the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of the TcRζ and CD3 chains (2–4). Phosphorylated ITAMs allow for ZAP-70 recruitment by means of tandem SH2 domain binding (5, 6). Activation of ZAP-70 catalytic activity then requires phosphorylation of the kinase at residue Tyr-493 by p56Lck (7, 8). The p56Lck SH2 domain can also bind to ZAP-70, thereby consolidating CD4-p56Lck within the TcR aggregate (9). The importance of p56Lck and ZAP-70 has been shown in kinase negative Jurkat cells that show defects in Ca2+ mobilization, tyrosine phosphorylation, and IL-2 transcription (10, 11).

Recent studies have identified an array of immune cell-specific adaptor proteins in T-cells that act as substrates for upstream kinases and play important roles in T-cell function (12, 13). These include the LAT (linker for activation of T-cells), VAV, SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa), and FYB/SLAP (Fyn T-binding protein/SLP-76-associated protein)). LAT (pp36) is a transmembrane type III surface protein with a small extracellular region attached to a long cytoplasmic tail (14, 15). It is expressed exclusively in hematopoietic cells, primarily in T-cells, natural killer cells, mast cells but not B-cells. Rather than binding extracellular ligands, LAT has the hallmarks of a specialized surface protein that acts as an anchor for multiple intracellular proteins. LAT-binding proteins include the established transducing molecules such as growth factor binding protein-2 (GRB-2), Son of sevenless (SOS), VAV, phosphatidylinositol 3-kinase, phospholipase Cγ, the cellular homologue of Casitas B-lineage lymphoma protein (c-Cbl), and SLP-76. Consensus motifs for direct SH2 domain binding exist for GRB-2 and phospholipase Cγ, whereas other components may be indirectly recruited. Although the exact mechanism by which LAT is coupled to the TcRζ/CD3 complex remains to be defined, it is involved in integrating signals from the receptor as demonstrated by the ability of dominant negative form of LAT to inhibit NF-AT transcriptional activity (15). LAT is also needed for the activation of phospholipase Cγ and the Ras pathway (16).

One potentially important link to LAT is SLP-76, another immune cell-specific cytoplasmic adaptor (17). It is a relatively hydrophilic protein with an acidic amino-terminal region, several tyrosine consensus motifs, a central proline-rich region, and a carboxyl-terminal SH2 domain. As expected of an adaptor, SLP-76 also binds multiple proteins including Nck, c-Cbl, phospholipase Cγ, and VAV. SLP-76−/− knock-out mice show that SLP-76 is required for pre-TcR signaling with a block at the double negative stage of differentiation (18, 19). Jurkat T-cells lacking SLP-76 also show defects in Ras activation, CD69 expression, and the activation of NFAT transcriptional activity of the IL-2 promoter (20). SLP-76 serves as a substrate for ZAP-70 (21, 22), an event that facilitates binding to the SH2 domain of VAV (22–25). SYK also promotes the binding of the adaptor NCK to the same region, leading to the formation of a SLP-76-VAV-NCK trimolecular complex that may regulate cytoskeletal organization (26). Consistent with this, overexpression of SLP-76, NCK, and VAV enhanced TeR-induced actin polymerization, whereas dominant negative forms of these components inhibited polymerization (26).

SLP-76 is also connected with two other lymphoid-specific proteins, Vav and FYB/SLAP. Vav has a guanine nucleotide exchange factor domain for the Rho and Rac GTPases (27), a pleckstrin homology domain, a cysteine-rich (Cys-rich) domain, as well as two Src homology 3 (SH3) domains and a Src homology 2 (SH2) domain at its carboxyl terminus (28). The regulation of Rac is in turn correlated with defects in actin-dependent TeRζ/CD3 cap formation in Vav−/− T-cells (29, 30). VAV becomes tyrosine-phosphorylated in response to antigen-receptor ligation (31) and binds via its SH2 domain to SLP-76 (23–25).
Overexpression of VAV and SLP-76 increases TcR-mediated IL-2 transcription in a cooperative manner (23, 25), although VAV-SLP-76 complex formation itself is not needed for TcR-induced IL-2 production in all T-cells (22).

Another connection to SLP-76 signaling involves its SH2 domain binding to FYB/SLAP. FYB/SLAP was independently cloned on the basis of its ability to bind to the Src-related kinase FYN-T and SLP-76 (32, 33). It also has the hallmarks of an adaptor protein with several proline-rich regions, multiple tyrosine-containing motifs, and two putative nuclear localization sequences linked to a carboxyl-terminal SH3 domain. As with LAT and SLP-76, FYB is restricted in expression to lymphoid cells, in particular T-cells and macrophages, but not in B-cells. Two FYB isoforms at 120 and 130 kDa are known, which differ due to a 46-amino acid insert in the 130-kDa form between the two nuclear localization sequences. FYB also undergoes tyrosine phosphorylation in response to TcR ligation, an event diminished in FYN-T-deficient T-cells (35). However, unlike with SLP-76, transfection studies with FYB have yielded conflicting results on the role of FYB/SLAP in the regulation of IL-2 production. One report found that FYB/SLAP potentiated IL-2 production (33), and another report has postulated a negative function for the protein (32).

Part of the uncertainty related to the effects of FYB/SLAP on lymphokine production may be related to its binding to the Src kinase FYN-T, an immune cell-specific variant of the Src kinase FYN. FYN-T associates with the antigen-receptor in T-cells (36–39) and acts as a weak substitute for LCK in the phosphorylation of TcR ITAMs (6, 22). Although FYN–/– mice exhibit normal thymic development (40, 41), the loss of FYN in LCK–/– mice contributes to the block at the double negative stage of thymic development (42, 43). In addition, expression of constitutively active FYN in LCK–/– mice can restore the differentiation of single positive thymocytes (42). However, the selective binding of the SH2 domain of FYN-T (and not the other Src kinase LCK) to FYB provides a novel avenue by which the FYN-T kinase could intervene in TcR signaling (33, 44).

In this paper, we demonstrate that the Src kinase FYN-T (but not LCK or ZAP-70) regulates the binding of the FYB and SLP-76 SH2 domains to distinct sites on FYB. Furthermore, we show that members of the matrix co-localize in the cytoplasm of cells and cooperatively up-regulate TcR-initiated IL-2 promoter activity.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Spodoptera frugiperda (SF) cell line IPLB-SF21 was obtained from Invitrogen and was propagated as a monolayer culture in SF900 insect medium (Life Technologies, Inc.) supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamycin according to the procedure of Brown and Faulkner (60). Viral infections were performed at a multiplicity of infection of 5 for protein production and of 0.1 for virus production. COS cells were maintained in RPMI 1640 medium supplemented with 5% (v/v) FCS, 1% (w/v) penicillin and streptomycin, and 1 (v/v) L-glutamine. COS cells were transfected with cDNAs inserted into the SRA2 expression vector (gift of Dr. M. Streuli, Dana Farber Cancer Institute, Boston). COS cell transfections were conducted according to standard protocols. The murine T-cell hybridoma, DC27.10 (gift of Dr. R. Zamorska, Medical Research Council, London, UK), Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS and 1% (w/v) penicillin/streptomycin at 37 °C in an atmosphere containing 5% CO₂.

mAb to SLP-76 was kindly provided by Dr. Paul R. Findell (Syntex, Palo Alto, CA). Anti-phosphotyrosine mAb 4G10 was kindly provided by Dr. Tom Roberts (Dana Farber Cancer Institute, Boston). Anti-FYN mAb was purchased from Transduction Laboratories (Lexington, KY). Rabbit antisera against p59Fyn-T and p56Lck were generated against synthetic peptides corresponding to residues 35–51 and 39–64, respectively. Anti-murine CD3 (2C11) was obtained from American Type Culture Collection.

Immunoprecipitation and Immunoblotting—Immunoprecipitations were conducted as described previously (22, 61). Briefly, either 15 × 10⁶ SF21 cells were infected with the baculovirus encoding the different proteins, 2 × 10⁶ COS cells were transfected with the different DNAs using DEAE-dextran, or 25 × 10⁶ DC27.10 were electroporated with the different DNAs. After 2 days cells were harvested and lysed with 200 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin). Immunoprecipitation was carried out by incubation of the lysate with the antibody for 1 h at 4 °C, followed by incubation with 50 μl of protein A-Sepharose beads (10% (v/v)) for 1 h at 4 °C. Immunoprecipitates were washed 3 times with ice-cold lysis buffer and subjected to SDS-PAGE. For immunoblotting the immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose filters (Schleicher & Schuell). Filters were blocked with 5% (v/v) skim milk for 1 h in Tris-buffered saline, pH 8.0, and then probed with the indicated antibody. Bound antibody was revealed with horseradish peroxidase-conjugated rabbit anti-mouse or donkey anti-rabbit antibodies using enhanced chemiluminescence (ECL, Amersham Pharma Biotech).

Peptide Binding Analysis—Peptides were synthesized and high pressure liquid chromatography purified by the Molecular Biology Core Facility (Dana Farber Cancer Institute, Boston). The sequences of the peptides used were as follows (with pY indicating the phosphorylated residue): phosphorylated peptide III, DDDpYDGEEED; unphosphorylated peptide III, DDDYGGEED; phosphorylated peptide II, DEpY-DDVTS; unphosphorylated peptide II, DEVYDDVTS; phosphorylated peptide I, RGSYGYKTTA; unphosphorylated peptide I, RGYGYKTTA. For the binding analysis, peptides were coupled to AminoLink Plus gel beads ( Pierce). The beads were added to the lystate and incubated for 2 h at 4 °C. The precipitates were then washed 3 times with ice-cold lysis buffer and subjected to SDS-PAGE and immunoblotting.

Anti-TcR Ligation Studies—To activate Jurkat and the DC27.10 murine T-cell hybridomas, 50 × 10⁶ cells were incubated with prewarmed RPMI media supplemented with 2% FCS, containing either 1 μg/ml OKT3 and 2 μg/ml anti-mouse for Jurkat or 5 μg/ml anti-CD3e (anti-CD3) and 10 μg/ml of the rabbit anti-hamster antibody for DC27.10 at 37 °C for varying lengths of time. Following activation, cells were then rapidly pelleted and solubilized in lysis buffer, as described above.

Immunofluorescence—Cells were plated on glass coverslips and grown for 1 day prior to staining. Cells were rinsed with PBS, fixed with 2% paraformaldehyde in PBS for 10 min, and permeabilized in 0.5% (v/v) Triton X-100 in PBS. To detect FYN-T, FYB, and SLP-76, permeabilized cells were exposed to the antigen-specific antibodies in blocking buffer (2% (v/v) normal goat and 10% (v/v) rabbit serum in PBS) for 1 h, followed by a brief wash and incubation with isotype-specific secondary antibody (Texas Red or FITC-labeled, Southern Biotechnology Associates, Birmingham, AL) and 0.5 mg/ml Hoechst dye 33258 (Sigma, to visualize DNA (blue)). Slides were mounted in a polyvinyl alcohol medium and viewed on a Nikon FXA microscope equipped for epifluorescence. Photographs were taken using Fujichrome ASA 400 film.

IL-2 Luciferase Assay—Jurkat cells (2 × 10⁷) were co-transfected with 20 μg of FYN-T, FYB, and SLP-76 cDNAs alone or in combinations with 2 μg of IL-2 full-length LUC reporter plasmid or 3 μg NPA-TAP-LUC reporter plasmid (kindly provided by Dr. Burakoff, Dana Farber Cancer Institute, Boston) and 0.2 μg of a control reporter plasmid (pRL-TK from Promega). Cells were pulsed using BTX Gene Pulser at 250 V, 800 microfarads in 10% FCS. Cells (1 × 10⁶) were aliquoted into a 12-well plate 16 h after transfection and cultured in a final volume of 1 ml of RPMI growth medium. Cells were unstimulated or stimulated at 37 °C with OKT3 or 10 ng/ml PMA. After 6 h stimulation cells were lysed in 100 μl of lysis buffer (Promega kit). Luciferase activity was determined using the luminesometer (MicroLumat, EG&G Berthold) immediately after the addition of 100 μl of luciferase substrate (Promega kit) followed by a Stop and Go reaction to measure the control reporter plasmid (dual luciferase system kit from Promega). Luciferase units of the experimental vector were normalized to the level of the control vector in each sample.

RESULTS

FYN-T Mediates FYB Phosphorylation—Previous studies have shown that FYB preferentially associates with the Src

---

2 M. Veale, M. Raab, Z. Li, A. da Silva, S.-K. Krafft, S. Wernemovicz, C. C. Morton, and C. E. Rudd, submitted for publication.
kinase FYN-T and the intracellular signaling protein SLP-76 (32, 33, 35). Preliminary data also implicated FYN-T in the phosphorylation of FYB (33). To determine the specificity of FYN-T phosphorylation of FYB and to assess whether it regulates FYB-SLP-76 complex formation, FYB was co-expressed with either the LCK, FYN-T, or ZAP-70 kinase in COS cells and was assessed for phosphorylation by anti-phosphotyrosine blotting (Fig. 1A). Only FYN-T was found to consistently phosphorylate FYB (lane 3). ZAP-70 failed to phosphorylate the protein in each of five experiments (lane 4). Similar negative results were obtained using the combination of LCK and ZAP-70 (data not shown). In addition, LCK showed little phosphorylation of FYB (lane 2). Specificity was also seen in a comparison with SLP-76 as substrate (Fig. 1B). In this case, as previously reported (21, 22), ZAP-70 preferentially phosphorylated SLP-76 (lane 4). Some FYN-T phosphorylation of SLP-76 was observed but at levels some 2–5-fold less than ZAP-70 (lane 3). No phosphorylation by LCK was evident (lane 2), although using the same system, LCK has previously been found to phosphorylate selectively the TcR ζ chain (6, 22). As an internal control for expression, FYB and SLP-76 (Fig. 1, A and B, lower panels), and the kinases (Lck, FYN-T, and ZAP-70) in each co-transfected construct expressed at significant levels (data not shown).

These observations demonstrate that among the three TcR associated kinases, FYN-T preferentially phosphorylates FYB.

**FYN-T Phosphorylation Provides Conditions for FYN-T Binding**—In order for FYN-T phosphorylation of FYB to be of functional importance, one would expect the kinase to phosphorylate sites that are needed for FYN-T and/or SLP-76 SH2 domain binding. To examine this, it was first necessary to identify the site(s) of FYN-T phosphorylation in FYB. To accomplish this, several deletion mutants of FYB were generated from residues 450, 585, 670, and 707 to the carboxyl terminus (Fig. 1). Only FYN-T was found to consistently phosphorylate FYB (lane 3). ZAP-70 failed to phosphorylate the protein in each of five experiments (lane 4). Similar negative results were obtained using the combination of LCK and ZAP-70 (data not shown). In addition, LCK showed little phosphorylation of FYB (lane 2). Specificity was also seen in a comparison with SLP-76 as substrate (Fig. 1B). In this case, as previously reported (21, 22), ZAP-70 preferentially phosphorylated SLP-76 (lane 4). Some FYN-T phosphorylation of SLP-76 was observed but at levels some 2–5-fold less than ZAP-70 (lane 3). No phosphorylation by LCK was evident (lane 2), although using the same system, LCK has previously been found to phosphorylate selectively the TcR ζ chain (6, 22). As an internal control for expression, FYB and SLP-76 (Fig. 1, A and B, lower panels), and the kinases (Lck, FYN-T, and ZAP-70) in each co-transfected construct expressed at significant levels (data not shown). These observations demonstrate that among the three TcR associated kinases, FYN-T preferentially phosphorylates FYB.

When co-expressed with FYN-T and the deletion mutants were subjected to precipitation using GST-SH2 fusion proteins. Under these conditions, FYN-T phosphorylation of FYB was found to induce the binding of the SH2 domains of both FYN-T and SLP-76 (Fig. 2A, lanes 2 and 8 versus 1 and 7). Without FYN-T co-expression, no SH2 domain precipitation occurred (lanes 1 and 7). Furthermore, in keeping with the previously outlined phosphorylation experiments, SH2 domain binding of both proteins was completely lost with the deletion of the region between residues 585 and 670 (lanes 4 versus 5 and 10 versus 11).

To demonstrate direct binding, the same experiments were performed by western protein-protein blotting. In this case, cell lysates were transferred to nitrocellulose and blotted with the individual GST-SH2 fusion proteins, followed by detection with an anti-GST mAb. Under this regime, direct binding by the SH2 domain of FYN-T and SLP-76 required FYN-T co-expression (Fig. 2B, lanes 2 and 8 versus 1 and 7). In addition, SH2 domain binding was lost with the deletion of the region between residues 585 and 670 (lanes 4 versus 5 and 10 versus 11). GST alone failed to show binding (data not shown). These data make the important point that FYN-T phosphorylation of FYB in the region between residues 585 and 670 is required for the binding of the SH2 domains of FYN-T and SLP-76.

Between residues 585 and 670, there exist only three potential tyrosine phosphorylation motifs (YDDV, residues 595–598; YDGI, residues 625–628; YDDV, residues 651–654) (Fig. 3A). The FYN-T and SLP-76 SH2 domains could bind to the same or different sites. Of these sites, the YDGI motif most closely resembles the optimal SH2-binding site for Src-related kinases YEEI (45). The expected motif for the SLP-76 SH2 domain has not been clearly established from peptide binding studies. To test this motif for binding, phosphorylated and non-phosphorylated peptides corresponding to this site (DDY{DYDGHEEED}) (peptide III) were coupled to AminoLink Plus beads and used to precipitate antigen from cell lysates, followed by anti-FYN and anti-SLP-76 immunoblotting. Anti-Lck was included as a control. Significantly, the Y{DYDGHEEED} peptide precipitated FYN-T (lanes 3–5). Precipitation occurred in a concentration-dependent fashion that was correlated with the amount of peptide (10, 20, and 40 µg) (middle panel, lanes 3–5). The nonphosphorylated peptide failed to precipitate detectable protein (lane 2). By contrast, phosphorylated peptide DDDYDGHEEED failed to precipitate Lck (lanes 8–10) and precipitated only negligible amounts of SLP-76 (lanes 13–15).

**In vivo studies confirmed in vitro binding data with the expression of a single site YDGI mutant (phenylalanine substituted for tyrosine) followed by analysis of SH2 domain binding (Fig. 3B). Under these conditions, GST-SH2 domain precipitated significantly less of the FDGI mutant than wild-type FYB (lane 2 versus 1). The mutant was generally expressed at

---

3 Z. Songyang and L. C. Cantley, personal communication.
FIG. 1. FYN-T phosphorylates FYB between residues 585 and 670. A, differential phosphorylation of FYB by the p56<sup>Lck</sup>, p59<sup>FYN</sup>-T, and ZAP-70 kinases. HA-tagged FYB was either expressed alone (lane 1) or in the presence of LCK (lane 2), FYN-T (lane 3), or ZAP-70 (lane 4) in COS cells. Lysates were prepared in 1% Triton X-100 lysis buffer and immunoblotted with an anti-Tyr(P) antibody.

B, differential phosphorylation of SLP-76 by the p56<sup>Lck</sup>, p59<sup>FYN</sup>-T, and ZAP-70 kinases. SLP-76 was either expressed alone (lane 1) or in the presence of LCK (lane 2), FYN-T (lane 3), or ZAP-70 (lane 4) in 293 cells. Lysates were prepared in 1% Triton X-100 lysis buffer, immunoprecipitated with anti-SLP-76 antibody (lanes 1–4), and immunoblotted with an anti-Tyr(P) antibody. Equivalent levels of FYB or SLP-76, respectively, were present in all lanes as demonstrated by immunoblotting with an anti-HA mAb or anti-SLP-76 mAb, respectively (lower panel). Densitometric analysis using the Scanjet laser scanner (Hewlett-Packard) of anti-Tyr(P) binding to FYB (A) or SLP-76 (B) positions of molecular mass markers (kDa) are indicated.

C, phosphorylation maps to a region between residues 585 and 670. Upper panel, representation depicts overall structure and the location of potential motifs that include a proline-rich region followed by two putative lysine/glutamic acid/arginine-rich nuclear localization motifs, an SH3-like domain, and several potential phosphotyrosine-based motifs. Deletion mutants of FYB were generated, from 450, 585, 670, and 707 to the carboxyl terminus. Middle and lower left panels, lysates from COS cells transfected with HA-FYB alone (lane 1) or combination of FYN-T (lane 2), deletion mutants HA-707 (lane 3), HA-670 (lane 4), HA-585 (lane 5), or HA-450 (lane 6) were prepared and subjected to immunoblotting with anti-Tyr(P) antibody (upper left panel) or anti-HA antibody (lower left panel). Middle and lower right panels, FYN-T phosphorlates FYB between residues 670 and 585 in T-cells. DC27.10 cells were transfected with Mock (lane 1), HA-FYB (lane 2), HA-FYB Δ707 (lane 3), HA-FYB Δ670 (lane 4), HA-FYB Δ585 (lane 5), HA-FYB Δ450 (lane 6), wild type (WT) (lane 7). HA-FYB Δ707, Δ670, Δ585, and Δ450, and WT were co-transfected with FYN-T (lanes 3–7). Upper right panel, anti-Tyr(P) blot; lower right panel, anti-HA blot. D, FYN-T does not phosphorylate FYB at residue 472. COS cells transfected with a GST-FYB clone (termed J12) comprises the residues 395–545 (lane 1), or co-transfected with FYN-T (lane 2), mutant form of J12 with an exchange of tyrosine to phenylalanine (lane 3) or WT-FYB, were lysed and immunoblotted with anti-Tyr(P) antibody (lanes 1–4, upper panel) or anti-glutathione S-transferase (GST) antibody (lower panel).
FIG. 2. FYN-T regulates FYN-T and SLP-76 SH2 domain binding to a region between FYB residues 585 and 670. A, COS cells were transfected with HA-FYB (lanes 1 and 7), HA-FYB/FYN-T (lanes 2 and 8), deletion mutant HA-707/FYN-T (lanes 3 and 9), HA-670/FYN-T (lanes 4 and 10), HA-585/FYN-T (lanes 5 and 11), or HA-450/FYN-T (lanes 6 and 12), lysed in 1% Triton X-100, and subjected to precipitation with GST-FYN-T SH2 (lanes 1–6) and GST-SLP-76 SH2 (lanes 7–12). The precipitates were separated on an SDS-7.5% polyacrylamide gel and subjected to anti-HA blotting. B, lysates from cells transfected with HA-FYB (lanes 1 and 7), HA-FYB/FYN-T (lanes 2 and 8), and deletion mutant HA-707/FYN-T (lanes 3 and 9). HA-670/FYN-T (lanes 4 and 10), HA-585/FYN-T (lanes 5 and 11), and HA-450/FYN-T (lanes 6 and 12) were separated on an SDS-7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The nitrocellulose membrane was incubated with anti-GST-SH2 FYN-T (lanes 1–6) or anti-GST-SH2 SLP-76 (lanes 7–12) followed by anti-glutathione S-transferase (GST) blotting.

within the 585–670 region, two remaining YDDV motifs at residues 595 and 651 exist that could bind to SLP-76. The two EVY₁⁵₁DDV and EVY₁⁶₁DDV motifs are similar, each containing conserved EV residues preceding the YDDV sequence (Fig. 3C). For our analysis, we chose to examine the carboxyl-terminal YDDV motif. Beads attached to phosphorylated DEVY₁⁶₁DDVDTSD peptide (peptide IV) precipitated SLP-76 (upper panel, lane 6). The non-phosphorylated peptide did not precipitate the protein (upper panel, lane 5). Moreover, the motif DDVY₁⁵₁DGIEEED (peptide III, residues 625–628) that binds to the FYH SH2 domain failed to precipitate SLP-76 (upper panel, lanes 2–4). Conversely, the SLP-76 binding motif failed to bind to the FYH SH2 domain (upper panel, lanes 9–11). As an additional control, another motif within RGSY₁⁵⁰GYKTTA (peptide I, residues 559–562) did not precipitate the SLP-76 protein (upper panel, lanes 7 and 8). The binding of phosphorylated DEVY₁⁶₁DDVDTSD peptide to SLP-76 was confirmed in Jurkat and DC27.10 T-cells (lower panel, lanes 4 and 12, respectively). Although these later data will require confirmation by site-directed mutagenesis, they document SLP-76 SH2 domain binding to YDDV motif of FYB.

FYB-FYN-SLP-76 Co-localize in Perinuclear Areas—Given our demonstration of a FYN-T-regulated matrix involving FYN-T and SLP-76 binding to FYB, it was next of interest to assess whether the proteins co-localized in cells. Immunofluorescence microscopy studies were performed using transfected COS cells and a combination of FITC and Texas Red-labeled secondary antibodies. Under these conditions, FYN was found principally in the cytoplasm of the cell and in particular around the nucleus (Fig. 4A, upper left panel). Perinuclear staining became more diffuse with an increasing distance from the nucleus. Minimal staining was observed at the cell surface. In more distal regions, FYB was also occasionally found in dot-like vesicular structures. Significantly, this pattern was also observed for FYN-T (Fig. 4A, upper right panel). Overlapping of the two images (FYB (FITC, green) and FYN-T (Texas Red, red)) revealed extensive co-localization with visible yellow-orange fluorescence that is characteristic of coincident staining (Fig. 4A, lower left panel). Co-localization was extensive and almost complete (i.e., there were few regions where FYB was found without FYN-T, and vice versa). Hoechst staining was used to identify the nucleus in both stained and unstained cells (Fig. 4A, lower right panel). The lack of staining in non-transfected cells served as a fluorescence negative control. As a further control, the FYB staining was contrasted with the staining pattern for the lysosomal marker LAMP-2. As described previously (46), LAMP-2 showed the presence of discrete spots, as well as clustering in the poles next to nuclei (Fig. 4C, right panel). LAMP-2 and FYB showed a partial intense co-localization in the polar cluster but not in the other areas of the cell stained for FYB (i.e., red color) (Fig. 4C, left panel). Taken together, these observations demonstrate a remarkably extensive in vivo co-localization of FYN-T and FYB in cells.

Given this result, we also investigated FYB and SLP-76 localization in COS cells. As shown in Fig. 4B, SLP-76 was also detected concentrated around the nucleus (upper right panel), coincident with FYB (upper left panel and lower left panel). Hoechst staining defined the position of the nuclei in these cells (Fig. 4B, lower right panel). Again, the degree of overlap was remarkably complete. The pattern was also distinct from LCK which was expressed almost exclusively on the cell surface (data not shown). Immunofluorescence in T-cells also showed extensive co-localization, but due to the small amounts of cytoplasm, it was not possible to carry out a more detailed analysis of its perinuclear or vesicular localization (data not shown). Together, the observation of FYN-FYB and FYN-SLP-76 co-localization is consistent with biochemical data showing a regulated interaction between the FYN-T, FYB, and SLP-76 proteins.

FYB-FYN-SLP-76 Exhibits Unusually Stable Phosphorylation—Previous studies have shown that FYB/SLP undergoes phosphorylation following TcR ligation (32, 35). With the observation that FYB and SLP-76 bind to each other and that this binding was regulated by FYN-T, the kinetics of phosphorylation was examined in the context of FYB-SLP-76 binding. As a control, TcR ligation resulted in a transient increase in tyrosine phosphorylation of the ζ chain over 5–20 min which was followed by a loss of phosphorylation from 30 to 60 min (Fig. 5A, lanes 1–8). This is typical of the tyrosine phosphorylation pattern described for other substrates such as the VAV and Cbl (31, 47, 48). In contrast, FYB showed remarkably stable phosphorylation, for as long as 24 h following T-cell receptor ligation (Fig. 5B, lanes 1–6). Similar kinetics of extended phosphorylation was observed for SLP-76 (lanes 1–6). Furthermore, as seen in the anti-SLP-76 precipitations, complex formation between FYB and SLP-76 was stable over the same time (lanes 2–6). These data indicate that the phosphorylation of FYB and
FIG. 3. FYN-T SH2 domains binds to YDG1 site in FYB. A, depiction of the FYB antigen: residues 622–632 comprise the peptide III including the YDG1 motif, residues 648–659 comprise the peptide II including the YDDV motif, and residues 556–566 comprise the peptide I including a YGYI motif. Lysates from COS cells expressing FYN-T, LCK, or SLP-76 were incubated with different amounts of Amino-Link Plus coupled non-phosphorylated peptide III (lanes 2, 7, and 12) and phosphorylated peptide III (lanes 3–5, 8–10, and 13–15). The precipitates were subjected to immunoblotting with anti-Fyn (lanes 1–5), anti-Lck (lanes 6–10), or anti-SLP antiserum (lanes 11–15). Lanes 1, 6, and 11 show positive control lysates from FYN-T (lane 1), LCK (lane 6), and SLP-76 (lane 11) expressing COS cells. B, COS cells were co-transfected with FYN-T and HA-FYB (lane 1) or with the single mutant HA-Y:FDGI of FYB (lane 2), lysed in 1% Triton X-100, and subjected to precipitation with GST-SH2 FYN-T (lanes 1 and 2). The precipitates were separated on an SDS-7.5% polyacrylamide gel and subjected to anti-HA blotting. Level of FYB expression is demonstrated by immunoblotting with an anti-HA mAb of lysates (middle panel). Densitometric analysis of FYB and FYB mutant binding to GST-SH2-FYN (right panel). C, upper panel, lysates from COS cells expressing SLP-76 or FYN-T were incubated with Amino-Link Plus coupled to non-phosphorylated peptide III (lane 2), phosphorylated peptide III (lanes 3 and 4), non-phosphorylated peptide IV (lanes 5 and 10), phosphorylated peptide IV (lanes 6 and 11), and phosphorylated peptide I (lanes 7 and 8). The precipitates were subjected to immunoblotting with anti-SLP-76 (lanes 2–8) or anti-Fyn (lanes 10 and 11). Lanes 1 and 9 show positive control lysates from SLP-76 (lane 1) and FYN-T (lane 9) expressing COS cells. Lower panel, lysates of Jurkat and DC27.10 cells were incubated with Amino-Link Plus coupled phosphorylated peptide III (lanes 2 and 10), peptide IV (lanes 4 and 12), peptide I (lanes 6 and 14), and phosphorylated peptide III (lanes 3 and 11), peptide IV (lanes 5 and 13), and peptide I (lanes 7 and 15). The precipitates were subjected to immunoblotting with anti-SLP-76 antiserum. As a positive control lanes 1 and 9 show the lysates of Jurkat and DC27.10, respectively, and lanes 8 and 16 show anti-SLP-76 immunoprecipitates.
SLP-76 and complex formation follows a kinetics that is distinct from previously observed TcR-induced tyrosine kinase substrates in T-cells.

**FYN-FYB-SLP-76 Cooperates in TCR-mediated IL-2 Gene Activation**—To address the functional significance of this pathway, FYN, FYB, and SLP-76 were co-expressed in Jurkat cells and assessed for an effect on the transcriptional activity of the IL-2 promoter (Fig. 6). Plate-bound anti-CD3 mAb was used at 0.1–1.0 μg/ml and caused a 2–4-fold stimulation of transcription in mock-transfected cells. Under these conditions, FYB overexpression had a little positive effect on the activity of the full-length promoter (Fig. 6A, left panel), as previously reported (35). Overexpression of SLP-76 alone led to moderate 2.5–7-fold increase in transcription, consistent with previous reports (23, 49). Wild-type FYN-T had a similar potentiating effect (Fig. 6A, left panel). By contrast, co-transfection of FYN-T and SLP-76 or FYB and SLP-76 resulted in the significant enhancement of transcription relative to the effect of the individual components. However, the most striking enhancement was observed with the combined expression of all three FYN-T, FYB, and SLP-76 proteins (Fig. 6B, right panel). In this case, a 30–50-fold amplification of TcR-driven IL-2 transcriptional activity was observed relative to the untransfected cells (right panel) (note different ordinate values between right and left panels).

The effect was synergistic relative to the effect of the individual components. Each component was expressed in the same vector using the same concentration of DNA, and each protein was expressed at equivalent levels (data not shown). Given that FYB acts as a scaffold for FYN-T and SLP-76 binding, it was next of interest to assess whether binding was required for the functional effect. Significantly, the Δ585 mutant of FYB that lacks the FYN/SLP-76-binding sites failed to potentiate IL-2 transcription (Fig. 6A). Under these conditions, the potentiating effect was reduced to the level observed with FYN/SLP-76 alone. These findings demonstrate that components of the FYN-FYB-SLP-76 pathway act in a cooperative manner to up-regulate IL-2 gene activation.

We next compared the level of synergistic activation by FYN-FYB-SLP-76 to that mediated by the LCK-ZAP-70 pathway using an NF-AT/AP-1 reporter construct (Fig. 6B, right panel). Whereas wild-type LCK and ZAP-70 alone had no potentiating effect, the combination of LCK and ZAP-70 increased transcription by some 10-fold, and the combination of LCK-ZAP-70-SLP-76 increased transcription by some 100–200-fold. These effects are in accord with the pathway where LCK activates ZAP-70 to phosphorylate SLP-76 (7, 8, 21, 22). As with FYN-FYB-SLP-76, it also indicates that the co-expression of the individual proteins is needed to observe the full potentiating effects. By comparison, co-transfection of FYN-T, FYB, and SLP-76 proteins caused a 50–100-fold amplification of NF-AT transcriptional activity relative to the untransfected cells (Fig. 6B). Perhaps not unexpectedly, the stimulatory effect of the LCK-ZAP-70-SLP-76 pathway on NF-AT transcriptional activity was generally 1.5–2-fold greater than that observed for FYN-FYB-SLP-76. Therefore, in both cases, the combined ex-

---

**Fig. 5. Stable phosphorylation of FYB and SLP-76 following TCR ligation.** A and B, DC27.10 cells were incubated with the 145-2C11 anti-CD3 e mAb at 37 °C for the times indicated. Cells were immediately solubilized with Nonidet P-40 lysis buffer. Lysates were incubated with either anti-CD3 (A) or anti-SLP-76 mAb (B) and subjected to immunoblotting with anti-Tyr(P) mAb.

**Fig. 4. Immunofluorescence co-localization of FYB with FYN-T and SLP-76.** COS cells transfected with FYB, FYN-T, and SLP-76 were stained with FITC-labeled or Texas Red-labeled antibody as described under “Experimental Procedures.” A, FYB and FYN-T show perinuclear co-localization. Anti-FYB, upper left panel; anti-FYN, upper right panel; anti-FYB and anti-FYN, middle left panel (dual exposure revealed co-localization of FYB and FYN-T (yellow fluorescence); Hoechst staining of nuclei, middle right panel; Hoechst staining of nuclei, lower right panel. C, FYB and Lamp-2 show different intracellular staining patterns. Anti-FYB and anti-LAMP-2, left panel; anti-LAMP-2, upper right panel.
expression of components of the FYN-FYB-SLP-76 or LCK-ZAP-70-SLP-76 pathways evoked potent synergistic up-regulation of IL-2 transcription.

**DISCUSSION**

TcR triggering initiates tyrosine phosphorylation of intracellular substrates required for lymphokine production and proliferation of T-cells. The exact nature and number of the protein-tyrosine kinases and substrates in the signaling pathways is not entirely understood. CD4/CD8-associated LCK initiated TcRz phosphorylation, and ZAP-70 recruitment is well established as an obligatory pathway in the activation process (2, 4). Recent studies have implicated additional downstream immune cell adaptors LAT, SLP-76, and FYB/SLAP in the activation process (15, 23, 25, 32, 33). LAT and SLP-76 act as substrates of ZAP-70 and SYK (15, 21, 22, 26) and are needed for TcR induction of IL-2 transcription (15, 20). The present model therefore connects the TcR with SLP-76 via ZAP-70 (SYK). By contrast, despite its binding to SLP-76, the mechanism by which FYB participates in signaling has been unclear. In this study, we describe a FYN-T-driven pathway where FYN-T, FYB, and SLP-76 form a matrix with FYB as a scaffold for FYN-T phosphorylation at sites that recruit FYN-T and SLP-76. Moreover, by successfully co-transfecting these three components in T-cells, we demonstrate that the FYN-T-FYB-SLP-76 pathway cooperatively up-regulates TcR-induced IL-2 transcription. Our observations add to the complexity of T-cell signaling by documenting the presence of a connection with SLP-76 that provides a novel avenue by which the FYN-T kinase can intervene in TCR signaling.

The FYN-T-FYB-SLP-76 pathway exhibited several distinguishing features. First, of the various TcR-associated kinases, only FYN-T was found to phosphorylate FYB (Figs. 1–3). This selectivity is unusual with only one other FYN-T-specific substrate having been described (50). Based on the use of deletion mutants, FYN-T phosphorylated FYB predominantly in a restricted region between residues 585 and 670, a region that carries binding sites for FYN-T and SLP-76 (Figs. 2 and 3). Within this region, two distinct but proximal tyrosine residues (Y625DGI and Y 651DDV) bound the FYN-T SH2 domain and SLP-76 SH2 domain, respectively. Peptide DDIY 625DGIEEED bound specifically to the FYN-T SH2 domain, without binding to the SLP-76 or LCK SH2 domains (Fig. 3). Similarly, a single site substitution of the tyrosine residue also reduced FYN-T SH2 domain binding in vivo (Fig. 3). Consistent with this, YDGI site possesses basic features of an Src SH2 domain-binding site, as observed in the optimal motif YEEI (45). FYN-T therefore acts to autoregulate its own binding to FYB, where it could then possibly use FYB as a scaffold to phosphorylate other sites and/or associated proteins. At separate sites, FYN-T and SLP-76 could act to send separate signals or, by virtue of their proximity, could cross-regulate and integrate their signaling. It is important to note that our analysis focuses on the FYN-T-regulated pathway. Since FYB shows some residual phosphorylation (some 40%) in FYN-T-deficient T-cells (35), it

![Fig. 6. FYN-T-FYB-SLP-76 potentiates TcR-induced IL-2 gene activation. A, Jurkat T-cells were subjected to extrapolation using 5 μg of full-length IL-2 promoter luciferase reporter plasmid and 0.2 μg of RL-TK plasmid together with either 20 μg of empty vector or various combinations of FYB, FYN-T, SLP-76, or Δ585, FYN-T and SLP-76 (see “Experimental Procedures”). Cells were either unstimulated (solid bars) or stimulated with either OKT3 (open bars) or OKT3 plus PMA (10 ng/ml) (hatched bars) for 6 h and assayed for luciferase activity. Luciferase units of the experimental vector were normalized to the level of the control vector in each samples. The data are representative of at least five independent experiments. Right and left panels represent points in the same experiment (note different ordinate values). B, Jurkat T-cells were co-transfected with 5 μg of IL-2 3XNFAT luciferase reporter plasmid and 0.2 μg of RL-TK plasmid together with either 20 μg of empty vector or various combinations of FYN-T, FYB, SLP-76, LCK, and ZAP-70. Cells were either unstimulated (solid bars) or stimulated with either OKT3 (open bars) or OKT3 plus PMA (10 ng/ml) (hatched bars) for 6 h and subsequently assayed for luciferase activity. Luciferase units of the experimental vector were normalized to the level of the control vector in each sample. The data are representative of at least five independent experiments.

FYN-T-FYB-SLP-76 Signaling Pathway

21177
also is clear that at least one other kinase exists in T-cells that can also phosphorylate the FYB adaptor. Our data therefore do not exclude the existence of other potential sites of SLP-76 or FYN-T SH2 binding mediated by other kinases in T-cells. Further studies are needed to uncover the full range of kinases capable of mediating phosphorylation and protein recruitment to the FYB scaffold.

In addition to their binding, FYN-T-FYB-SLP-76 was found to co-localize in the cytoplasm and perinuclear region of cells (Fig. 4). The degree of co-localization is remarkably complete, with most FYN-T co-localized with FYB and vice versa. Intra-cellular staining of FYN-T near centrioles has been reported by others (51). This pattern is different from that of the lysosomal marker Lamp 2 and of p56Lck which is localized at the cell surface (data not shown). ZAP-70 has recently been detected at both the cell surface and in the nuclear region of cells (52). This distinct localization could contribute topographically to the independence of the FYN-T-FYB-SLP-76 pathway, where discrete co-localization would facilitate phosphorylation and binding. In addition to their discrete localization, FYN-T-FYB-SLP-76 showed an unusually long term stability of phosphorylation following TCR ligation. Instead of being transiently phosphorylated and dephosphorylated as with most substrates, FYB phosphorylation and complex formation remained remarkably stable, for as long as 24 h (Fig. 5). The pattern fits with the extended kinetics of FYN-T activation following anti-CD3 ligation (53, 54). The kinetics also introduces the possibility that FYN-T-FYB-SLP-76 contributes to early and late stages of the T-cell response, possibly contributing to the long term production of IL-2.

Finally, the combination of FYN-T-FYB-SLP-76 was found to potentiate synergistically TCR-driven IL-2 transcription (Fig. 6). TCR-driven promoter activity was increased 30–50-fold relative to non-transfected cells in the case of the full-length promoter and by some 50–100-fold with the NF-AT/AP-1 reporter. The effect was much greater than the effect of SLP-76 or FYN-T alone, where a 2–7-fold stimulation index was observed (Fig. 6) (23, 49). By contrast, the transfection with FYB alone had a weak effect on transcription. Our data therefore help resolve the previous discrepancies using FYB in transfection studies (32, 33), by showing that the combination of components of the FYN-T-FYB-SLP-76 pathway are needed for optimal potentiation. Each component was expressed under the control of the same expression vector using equal amounts of DNA resulting in comparable levels of expression. The requirement for multiple components and the resultant synergy suggest that the relative abundance of each constituent may be important in eliciting potentiation. Hence, differences in the relative abundance of constituents of this signaling matrix in different cells could theoretically produce different outcomes, given that both LCK-ZAP-70 and FYN-T-FYB ultimately engage the central adaptor SLP-76. Finally, in support of a model where FYB acts as a central scaffold in the FYN-T-driven pathway, deletion of the FYN-T- and SLP-76-binding sites in FYB (Δ585) completely abrogated potentiation. FYB may therefore act as an adaptor or scaffold, bringing FYN-T and SLP-76 into a functional aggregate needed for the generation of intracellular signals.

Synergy was also observed using the combination of LCK, ZAP-70, and SLP-76 (Fig. 6B). Although LCK and ZAP-70 alone had no potentiating effect, the combination of LCK-ZAP-70-SLP-76 augmented NF-AT mediated transcription by some 100–200-fold. This fits with the well established pathway where LCK activates ZAP-70 which phosphorylates SLP-76 (7, 8, 21, 22). To our knowledge, perhaps due to technical limitations in expressing three components in cells, a former demonstration of this synergy has not been reported. FYN-T-FYB-SLP-76 potentiation at levels some 50% that observed for the well established LCK-ZAP-76 pathway confirms the significance of the FYB pathway in regulating IL-2 production.

Another interesting aspect of our study is that the potentiating effects were obtained using the non-transforming, wild-type forms of FYN-T and LCK. Previous studies have relied on the use of constitutively active forms of Src kinases for potentiating effects on lymphokine production (55–57). The need for the co-expression of multiple components of a given pathway provides a potential explanation for the inability of previous studies to observe significant functional effects with individual wild-type kinases. The need for co-expression is likely due to the limited presence or accessibility of the endogenous proteins in cells. The potentiating effect of FYN-T-FYB-SLP-76 was significantly greater than with the transforming version of FYN-T (Y528F) (data not shown).

The involvement of FYN-T in the FYN-T-FYB-SLP-76 pathway does not exclude an additional role for FYN-T (particularly, the cell-surface form of the kinase) in overlapping functions with LCK, such as in the phosphorylation of TcR ITAM motifs (6). However, the demonstration that FYN-T can cooperate with FYB and SLP-76 in regulating signals indicates that FYN-T can also play a more specialized role in the signaling cascade in T-cells. The exact relationship of the FYN-T-FYB-SLP-76 pathway to the LCK-ZAP-70-SLP-76 pathway remains to be determined. However, qualitative and quantitative gradations in the response of the T-cells to different peptide ligands could be related to the differential engagement of the two pathways. Since the LCK-ZAP-70 and FYN-T-FYB pathways both converge in their binding to SLP-76, the requirement for SLP-76 in pre-TcR and mature TcR signaling (18–19) could be linked to one or both pathways.

Finally, several previous reports have implicated FYN-T in the induction of anergy (58, 59). Although our data do not exclude a role for the kinase in this event, they clearly demonstrate that the FYN-T kinase can have a potent potentiating role as well. Further studies will be needed to determine the initial events that couple the TcR to the FYN-T-FYB-SLP-76 pathway and the degree to which the documented importance of SLP-76 is related by the LCK-ZAP-70-SLP-76 and FYN-T-FYB-SLP-76 pathways.

REFERENCES
1. Rudd, C. E., Janssen, O., Cai, Y.-C., da Silva, A. J., Raab, M. & Prasad, K. V. S. (1994) Immunol. Today 15, 229–232.
2. Weiss, A. & Littman, D. R. (1994) Cell 76, 263–274.
3. Mustelin, T. (1994) Immunity 1, 351–356.
4. Wange, R. L. & Samelson, L. E. (1996) Immunity 5, 197–205.
5. Chan, A. C., Iwashima, M., Turck, C. W. & Weiss, A. (1992) Cell 71, 649–662.
6. Iwashima, M., Irving, B. A., Van Oers, N. S. C., Chan, A. C. & Weiss, A. (1994) Science 263, 1136–1139.
7. Chan, A. C., Dalton, M., Johnson, R., Kong, G. H., Wang, T., Thoma, R. & Kurokaki, T. (1995) EMBO J. 14, 2499–2508.
8. Wange, R. L., Guittain, R., Isakov, N., Watts, J. D., Aebersold, R. & Samelson, L. E. (1995) J. Biol. Chem. 270, 18773–18783.
9. Duplay, P., Thome, M., Herve, F. & Acuto, O. (1994) J. Exp. Med. 179, 1163–1172.
10. Strauss, D. & Weiss, A. (1992) Cell 70, 585–593.
11. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R., Samelson, L. M., Leibson, P. J. & Abraham, T. R. (1998) Mol. Cell. Biol. 18, 1388–1399.
12. Rudd, C. E. (1999) Cell 96, 1–20.
13. Peterson, E. J., Clements, J. L., Fang, N. & Koretzky, G. A. (1998) Curr. Opin. Immunol. 10, 357–344.
14. Weber, J. R., Orstavik, S., Torgerson, K. M., Danbolt, N. C., Berg, S. F., Ryan, J. C., Tasken, R., Imhoden, J. B. & Vaage, J. T. (1998) J. Exp. Med. 187, 1157–1161.
15. Zhang, W. J., Sloan-Lancaster, J., Kitchen, J., Trible, R. P. & Samelson, L. E. (1998) Cell 92, 83–92.
16. Finco, T. S., Kadlecek, T., Zhang, W., Samelson, L. E. & Weiss, A. (1998) Immunity 9, 617–626.
17. Jackman, J. K., Motto, D. G., Sun, Q., Tanemoto, M., Turck, C. W., Peitz, G. A., Koretzky, G. A. & Findell, P. R. (1995) J. Biol. Chem. 270, 7029–7032.
18. Clements, J. L., Yang, B., Ross-Barta, S. E., Eliason, S. L., Hirstka, R. F., Williamson, R. A. & Koretzky, G. A. (1998) Science 281, 416–419.
19. Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W. & Geha, R. S.
