Novel Isoform of Lymphoid Adaptor FYN-T-binding Protein (FYB-130) Interacts with SLP-76 and Up-regulates Interleukin 2 Production* 

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T-cell activation involves the participation of protein-tyrosine kinases p56lck and ZAP-70/SYK as well as lymphoid proteins such as SLP-76 and FYB/SLAP. FYB/SLAP has the hallmarks of an adaptor protein that binds to the SH2 domains of the Src kinase FYN-T and SLP-76. Whereas two forms of FYB at 120 and 130 kDa have been identified biochemically, a cDNA encoding only the lower molecular weight isoform has been cloned (termed FYB-120 or SLAP-130). In this study, we report the isolation of an alternative isoform of FYB with a molecular mass of 130 kDa (FYB-130) that has the same structure as FYB-120 except for an insertion of 46 amino acids toward the carboxyl-terminal region of the protein. FYB-120 and FYB-130 share an ability to bind to the SH2 domains of FYN-T and SLP-76, to act as substrates for p59FYNT, and to be expressed in the cytoplasm and nucleus of T-cells. Differences were noted between the isoforms in the efficiency of binding to SLP-76 and in the preferential expression of FYB-130 in mature T-cells. When co-expressed together with FYN-T and SLP-76, FYB-130 caused a significant increase in anti-CD3-driven NF-AT transcription. Finally, fluorescence in situ hybridization analysis localized the FYB gene to human chromosome 5 at position p13.1. FYB-130 therefore represents a novel variant of FYB protein that can up-regulate T-cell receptor-driven interleukin 2 production in mature T-cells.

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¶¶ The abbreviations used are: TcR, T-cell receptor; FYB, FYN-T-binding protein; SH, Src homology domain; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; IL-2, interleukin 2; GST, glutathione S-transferase; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; mAb, monoclonal antibody.

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also a hematopoietic protein with guanine nucleotide exchange factor activity for Rho and Rac GTPases (22). Overexpression of VAV and SLP-76 increases TcR-mediated IL-2 transcription in a cooperative manner (18, 20). However, binding per se between Vav and SLP-76 is not needed for TcR-driven production of IL-2 (26, 46).

An additional SLP-76 associated protein is the lymphoid-specific protein FYB/SLAP, a protein cloned on the basis of its ability to bind to the Src-related kinase FYN-T and SLP-76 (23, 24). FYB/SLAP 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/SLAP is expressed primarily in T-cells, monocytes, and mast cells but not in B-cells. FYB/SLAP also undergoes tyrosine phosphorylation in response to TcR ligation, an event diminished in FYN-T-deficient T-cells (25). Overexpression of FYB/SLAP alone in T-cells has been reported to either weakly potentiate (24) or inhibit IL-2 production (23). However, co-transfection of FYB/SLAP with its binding partners FYN-T and SLP-76 causes a potent up-regulation of TcR-driven IL-2 production (26). These data suggest that FYB plays a role in the integration of signals from the TcR complex.

Biochemical analysis of FYB has revealed two proteins FYB-120 and a higher Mr, form with an apparent mass of 130 kDa (termed FYB-130) (25). Isolated cDNAs for FYB or SLAP encode a protein that corresponds to the lower Mr, isoform. In order to establish the basis for the difference between FYB-120 and FYB-130, we re-screened a cDNA library to determine whether there exists an alternate form of FYB encoding the higher Mr, isoform. In this paper, we report the cDNA cloning of FYB-130 and demonstrate that this novel isoform binds to SH2 domains of FYN-T and SLP-76, localizes in the cytoplasm/nucleus of cells, and can potentiate TcR-driven IL-2 production in T-cells. FYB-130 differs from FYB-120 in its preferential expression in mature T-cells. FYB-130 represents a novel FYB isoform that positively regulates IL-2 production in mature T-cells.

### MATERIALS AND METHODS

**Cloning of FYB-130**—A murine T-cell hybridoma-derived cDNA library constructed into aZAP-Express (Stratagene, La Jolla, CA, generous gift of Dr. Linda Clayton, Dana Farber Cancer Institute, Boston) was screened with the human FYB cDNA under moderate stringency conditions. Plasmid DNA was purified from positive clones, and both strands were subjected to sequencing by automatic sequencer (Dana-Farber Cancer Institute Molecular Core Facility). DNA sequences were analyzed using the Genetics Computer Group (GCG) program (Madison, WI).

**Antibodies**—The generation of the anti-FYB serum, anti-FYN-T, has been previously described (27). Anti-HA for immunoblotting (12CA5) was obtained from the American Type Culture Collection. Rhodamine-labeled anti-mouse Ig was purchased from Caltag Laboratories.

**Protein Analysis**—Computer analysis of the FYB-130 protein sequence was conducted using the Prosite data base (28), and sequence homologies were analyzed using BLASTP and BEAUTY programs (29) and data bases available from the National Center for Biotechnology Information and Baylor College of Medicine. PEST sequences were based on prediction programs (30). Secondary structural analysis was conducted using the NNPREDICT program and the COILS program (31).

**Cells and cDNA**—COS cells and DC27.10 murine T-cell hybridoma cells were maintained in RPMI 1640 media supplemented with 5% fetal calf serum (v/v), 1% penicillin/streptomycin (v/v), and 1% L-glutamine (v/v) at 37 °C in an atmosphere containing 5% CO₂. COS cells were transfected with FYB-120 and FYB-130 using the SRα expression vector (gift of Dr. M. Streuli, Dana Farber Cancer Institute, Boston) and the pEB expression vector as described previously (24). Full-length FYB-120 or FYB-130 cDNAs were expressed in the pSRα or pEB mammalian expression vector carrying the influenza hemagglutinin (HA) epitope tag at the NH₂-terminal end (FYB-HA). GST-SH2 domain fusion protein for SLP-76 was kindly provided by Dr. Paul R. Findell (Syntex, Palo Alto, CA; GST-SH2 domain fusion protein for FYN-T has been previously described (24).

**Thymocyte T-cell Subset Purification**—Thymus or spleen cell suspensions were prepared, and thymocyte T-cell subsets were isolated using immunodepletion using anti-CD3 mAb (145-2C11, generous gift of Dr. Jeff Bluestone, University of Chicago, IL), anti-CD4, and anti-CD8 mAb-coated magnetic beads coated with anti-hamster and anti-mouse Ig (Magnetic Perspectives, Cambridge, MA).

**Immunofluorescence and Confocal Microscopy**—COS cells were seeded at a 1 × 10⁶ cells/well into 6-well culture dishes (Nunc, Denmark) containing 6 cm glass coverslips. After overnight incubation the cells were transfected with 2 μg of Srα-FYB-120- HA and pEB-FYB-130 HA DNA using Superfect (Qiagen). The cells were incubated for a further 48 h, and the coverslips were washed and removed and fixed in methanol for 5 min, for staining. The coverslips were washed in phosphate-buffered saline and incubated with anti-FYB rabbit serum, diluted 1:100 for 1 h at 37 °C. After further washing with phosphate-buffered saline FYB expression was detected after incubation with rhodamine-labeled secondary antibody, and nuclei were stained using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Eugene, OR; 0.5 μg/ml). Coverslips were mounted in glycerol gelatin (Sigma) onto microscope slides. Endogenous FYB expression was detected in T-cell hybridoma cells DC27.10 using anti-FYB rabbit serum. Immunofluorescence was analyzed using the confocal laser scanning microscope LSM 410 (Zeiss, Germany) equipped with an external argon-krypton laser (568 nm). The images were printed with the Fujix Pictography 3000 color printer (Fujifilm, Japan) using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

**Immunoprecipitations and Immunoblotting**—Precipitations were conducted using Triton X-100 lysis buffer (1% v/v in 20 mM Tris, pH 8.1; 150 mM NaCl), as described (32). Immuno blotting was carried out using chemiluminescence using the Renaissance detection kit (NEN Life Science Products), as described (32).

**NF-AT Promoter Luciferase Assay**—Jurkat cells (2 × 10⁵) were co-transfected with 20 μg of FYN-T, FYB, SLP-76 cDNAs alone or in combination and were aliquoted into a 12-well plate and cultured in a final volume of 1 ml of RPMI growth medium. Cells were unstimulated or stimulated at 37 °C with 0.1 ng/ml phorbol 12-myristate 13-acetate 16 h after transfection. After 6 h stimulation cells were lysed in 100 μl of lysis buffer (Promega kit). Luciferase activity was determined using the luminometer (MicroLucat, EGG7 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 samples.

**Fluorescence in Situ Hybridization**—Fluorescence in situ hybridization was conducted using probe labeling with digoxigenin: 1 μg of a 1.5-kDa human FYB cDNA cloned into the SRα vector was labeled with digoxigenin 11-dUTP as described in Zhao et al. (33), co-precipitated with 100 μg of rRNA and resuspended in 1× TE at 100 μg/ml. Fluorescence in situ hybridization, hybridization of metaphase chromosome preparations from peripheral blood lymphocytes obtained from normal human males was performed with the FYB gene at 10 μg/ml in Hybrisol VI according to previously described methods (34). Digoxigenin-labeled probe was detected using reagents supplied in the Oncor Kit (Oncor, Gaithersburg, MD) according to the manufacturer’s recommendations. Metaphase chromosomes were counterstained with DAPI. Map position of the human FYB was determined by visual inspection of the fluorescent signal on the DAPI-stained metaphase chromosomes. Twenty one metaphases were assessed for probe localization. Hybridization was observed with a Zeiss Axioshot microscope and images captured and printed using the Cytovision Imaging System (Applied Imaging, Pittsburgh, PA).

### RESULTS

**Isolation of FYB-130 Isoform**—The cloning of the gene encoding the lower Mr, form of FYB (or SLAP) has previously been described (23, 24). However, biochemical analyses have shown the presence of two bands at 120 and 130 kDa that do not appear to differ due to phosphorylation or glycosylation (25). In an attempt to isolate an alternative isoform of FYB encoding the 130-kDa isoform (FYB-130), we re-screened a murine cDNA library with oligonucleotide probes from FYB-120 that led to the identification of several positive clones. One new clone
encoded an isoform of FYB (FYB-130) that is identical to the 120-kDa isoform except for a 138-base pair insert (Fig. 1A). The FYB-130 insert (FYB-130i) encodes 46 amino acids that occurs at murine residue 627 and is located between two tyrosine-based motifs YDGI and YDDV (Fig. 1A). Secondary structure predictions indicate that the insert is helical between residues 637 and 648 (Fig. 1B). A charged grouping of residues enriched for lysine and aspartic acid (KGKDRKKK) precedes putative phosphorylation sites for the serine/threonine kinases, casein kinase II, protein kinase C, and cAMP or cGMP kinase. Two potential PEST sequences (polypeptide regions enriched in proline (P), glutamic acid (E), serine (S), and threonine (T) sequences), one of which is also found in the FYB-120 isoform, overlap with amino acids at the amino and the carboxyl termini of FYB-130i (Fig. 1B). Of potential significance, a comparison of FYB-130 with the protein data base shows significant homology with several nuclear proteins (Fig. 1C). These include the Asp/Glu-rich acidic site of HMG1/2-like protein (42% identical/68% similar from residues 641 to 669) (Fig. 1C), the hypothetical protein Mj1361 (41% identical/70% similar from residues 635 to 664), the myristoylation site for the DNA repair enzyme RecA (37% identical/68% similar from residues 628 to 662) (Fig. 1C), as well as the regulator of chromosome condensation in yeast (25% identical/55% similar from residues 628 to 670). The regions of homology extend over a significant portion of the 46 amino acid FYB-130i insert.

This new isoform also retains the structural features of murine FYB-120 that include type I (Arg-X-Pro-X-Pro) (residues 307–312) or type II (Pro-X-Pro-X-Pro-Ag/Lys) SH3 domain recognition motifs (residues 237–242, 357–362, and 417–422) (35), multiple tyrosine residues, two putative bipartite nuclear localization K/R/R/R-X,-X,-X,-X,-X,-X,-K/R/K motifs (residues 467–505 and 664–694 (FYB-120), 710–746 (FYB-130)) and an SH3-like domain found in FYB-120. Additional features of FYB-120 and -130 include consensus sites for serine/threonine kinases such as mitogen-activated protein kinase (36), protein kinase C, cAMP- or cGMP-dependent kinase, casein kinase II, and numerous possible myristoylation sites (Table I). Computer predictions of FYB secondary structure yielded a helical structure for the putative nuclear localization sequences that includes a coiled-coil (residues 458–493) and three potential PEST sequences for FYB-120 and four sequences for FYB-130 with possible phosphorylation sites for casein kinase II (Table I).

In order to verify that the new clone encoded a 130-kDa protein, FYB-130 tagged with HA was transfected into COS cells and analyzed for expression by anti-HA blotting (Fig. 2A, upper panel). The expressed protein migrated at approximately 130 kDa (lane 3), at a position on SDS-PAGE above the expressed FYB-120 protein (lane 1). Furthermore, FYB-130 co-migrated with the upper band of the FYB 120/130 doublet precipitated by anti-FYB from T-cell hybridoma DC27.10 (lane 5). DC27.10 is the T-cell hybridoma from which peptide sequence information was obtained for FYB (25).

We and others (23, 24) have previously shown that human FYB-120 is phosphorylated by FYN-T (25, 26) and interacts with FYN-T and SLP-76 SH2 domains. To assess whether FYB-130 shared this property, HA-tagged FYB-120 and FYB-130 were expressed individually with the FYN-T kinase and assessed for tyrosine phosphorylation by anti-phosphotyrosine immunoblotting (Fig. 2A, lower panel). COS cells were used to avoid competition from endogenous FYB in T-cells. Nevertheless, similar results were also obtained in the T-cell hybridoma DC27.10 (data not shown). Under these conditions, co-expression with FYN-T resulted in the detection of a highly phosphorylated FYB-130 band (lane 4), whereas FYB-130 alone showed no background phosphorylation (lane 3). Specificity was shown by co-expression of another kinase ZAP-70 that failed to phosphorylate FYB-130 (data not shown). Similar levels of FYB-120 phosphorylation by FYN-T were observed (lane 2 versus lane 4). Probing the same blot with anti-HA revealed that equivalent amounts of the FYB proteins had been expressed (upper panel, lanes 2 and 4). These observations indicate that like FYB-120, FYB-130 acts as a substrate for the FYN-T kinase.

In order to assess whether the SH2 domains of FYN-T and SLP-76 could bind to FYB-130, GST fusion proteins carrying the SH2 domains of FYN-T, SLP-76, or of the p85 subunit of phosphatidylinositol 3-kinase were added to cell lysates and assessed for their ability to precipitate FYB. As shown in Fig. 2B, c-expression of FYB-130 and FYN-T created conditions for the binding of FYB-130 to the SH2 domains of FYN-T and SLP-76 (lanes 8 and 12, respectively). In contrast, no binding was noted against singly expressed unphosphorylated FYB-130 (lanes 7 and 11, respectively). Specificity was shown by the inability of the SH2 domain of p85 to precipitate the protein (lanes 15–16, respectively). As an additional control, GST alone did not precipitate FYB-130 (lanes 3 and 4). From this, it is evident that FYB-120 and FYB-130 share the ability to bind to the SH2 domains of FYN-T and SLP-76. The only difference noted between FYB-130 and FYB-120 was in the efficiency of precipitation. Despite similar levels of FYB-130 and FYB-120 expression and phosphorylation, FYN-T and SLP-76 SH2 domain generally was found to precipitate greater amounts of FYB-120 (lanes 6 versus 8 and 10 versus 12, respectively).

FYB-120 and FYB-130 Expression in the Cytoplasm and Nucleus—Given the structural similarity and differences of the FYB isoforms, it was next of interest to assess whether FYB-120 and FYB-130 localized to similar regions in the cells (Fig. 3). Transfection of COS cells with either isoform of FYB-HA was detected with anti-FYB rabbit serum and rhodamine-labeled secondary antibody. Anti-FYB showed the presence of FYB-120 (Fig. 3a) and FYB-130 (Fig. 3b) in the cytoplasm and in the nucleus of the cells. Nuclear staining excluded the nuclei. A similar pattern of staining was also observed in cells stained with anti-HA mAb (data not shown). The specificity of the antibody staining is shown by negative staining in the non-transfected cells that are clearly identified in the field by DAPI staining of the nuclei (right panels a‘ and b’). Endogenous FYB was also significantly detected in the nucleus of the T-cell hybridoma DC27.10 (Fig. 3, d and d’). In this case, the cytoplasmic and nuclear staining pattern in DC27.10 T-cell hybridoma cells showed an even more pronounced dot-like pattern. As a negative control, when DC27.10 cells were labeled with secondary antibody alone (Fig. 3, c and c’), no fluorescence was observed, indicating specific labeling with the FYB anti- serum. These data clearly demonstrate the presence of FYB in the nucleus and cytoplasm of transfected COS and T-cells.

Differential Expression of FYB Isoforms in Thymus and T-cells—Given the restricted pattern of FYB expression to hematopoietic cells, it was of interest to assess whether FYB-120 and FYB-130 might be differentially expressed in thymocytes and mature T-cells. As seen in Fig. 4B, expression of FYB was detected in freshly isolated thymocytes and splenic derived mature T-cells (lanes 3 and 5, respectively). However, a difference in the relative abundance of the isoforms was noted where FYB-120 was the more abundant form in thymocytes (lane 3), and FYB-130 was expressed at higher levels in mature T-cells (lane 5). FYB-130 was generally represented at some 50–60% of that observed for FYB-120 in thymocytes (Fig. 4C, lower panel). These relative levels also varied from experiment to experiment where in some experiments little if any FYB-130 was detected in the thymocytes. In splenic T-cells, FYB-130 was generally present at twice the levels of FYB-120 (Fig. 4B, lower panel).
The prominence of the FYB-120 isoform was also apparent in populations of enriched immature thymocytes. Thymic differentiation follows a pattern from immature CD4+CD8−CD3+ cells to CD4+CD8+CD3− cells and finally to more mature CD4+CD8−CD3− or CD4−CD8+CD3− single positive thymocytes (37). Subpopulations were isolated by depletion using magnetic beads coated with either anti-CD3 or a combination of anti-CD4 and CD8 antibodies. As seen in Fig. 4A, depletion with anti-CD3 enriched for a population of immature CD4 and CD8 double-positive thymocytes (92.7%), whereas the combination of anti-CD4 and CD8 antibodies enriched for a population containing immature double-negative thymocytes (76.6%). In both immature subpopulations, FYB-130 was poorly represented relative to FYB-120 (lanes 1 and 2) (see histogram). Similarly, prothymocytic malignancy (CD4+CD8+) cells also expressed high levels of FYB-120 with little if any FYB-130 expression (lane 4). Finally, we and others (23, 24) have previously reported that the thymically derived T-cell line Jurkat expresses only the FYB-120 isoform. Together, these observations indicate that the relative expression of FYB isoforms differs in thymocytes and mature T-cells.

**FYB-120 and FYB-130 Up-regulate TcR Induction of IL-2 Expression**—The function of FYB-130 was also examined for an ability to up-regulate TcR-driven IL-2 production in cells expressing an NFAT reporter construct. We previously showed that the co-transfection of FYB-120 with its binding partners FYN-T and SLP-76 up-regulates TcR-driven IL-2 production (26). FYB-130 was therefore compared with FYB-120 in stimulation studies using plate-bound anti-CD3 mAb (0.1–1.0 μg/ml). As seen in Fig. 5 (upper panel), expression of FYB-120 or FYB-130 by itself had little effect on the activity of the promoter. SLP-76 expression alone had a moderate potentiating effect, as described by others (18, 38). Significantly, co-expression of FYB-130 or FYB-120 with FYN-T and SLP-76 synergistically potentiated TcR-induced IL-2 transcription. IL-2 transcription was increased by some 100–200-fold relative to mock-transfected cells. Interestingly, FYB-130 was generally found to have a more potent effect on transcription than FYB-120. This was observed using combinations of FYB, FYN-T, and SLP or in limited combinations where FYB-130 or FYB-120 was co-expressed with FYN-T. In four of six experiments, FYB-130 provided a more potent signal in the amplification of transcription relative to FYB-120. To assess whether FYB-120 and FYB-130 could cooperate with each other in regulation of transcription, they were also co-expressed in Jurkat cells with FYN-T and SLP-76. This resulted in an increase in transcription but at levels similar to that produced by increasing the expression of FYB-120 or FYB-130 individually. This indicates that FYB-120 and FYB-130 do not synergize with each other in the regulation of transcription. Each protein was expressed in the same vector using the same concentration of DNA, and as expected protein was expressed at similar levels (Fig. 5, lower panel). These findings demonstrate that the FYB-130 isoform can up-regulate IL-2 gene activation in T-cells. Furthermore, this isoform could potentiate transcription more than FYB-120, providing a possible rationale for the increased expression of FYB-130 over FYB-120 in mature T-cells.

**FYB Gene Localizes to the Short Arm of Chromosome 5**—Next, it was of interest to determine the chromosome localization of the human FYB gene. Precise chromosomal assignment of the human FYB gene was accomplished using fluorescent *in situ* hybridization analysis with a probe of sequences common to the FYB-120 and -130 (Fig. 6). Map position was determined by visual inspection of the fluorescent hybridization signals on DAPI-stained chromosomes. In 18 of 21 metaphase preparations analyzed, hybridization signals were found to be present on the short arm of chromosome 5 in band

| Potential protein kinase phosphorylation sites | Amino acid residue | Amino acid consensus |
|----------------------------------------------|-------------------|----------------------|
| cAMP and cGMP                               | 565–568           | KKNX                 |
| Protein kinase C                            | 652–655           | RKK5                 |
|                                             | 17–19             | SSR                  |
|                                             | 34–36             | SRK                  |
|                                             | 59–61             | TTK                  |
|                                             | 89–91             | SPR                  |
|                                             | 114–116           | SPK                  |
|                                             | 212–214           | TSK                  |
|                                             | 754–756           | TSK                  |
|                                             | 329–331           | TPK                  |
|                                             | 455–457           | SGK                  |
|                                             | 518–520           | SFP                  |
|                                             | 542–544           | TAR                  |
|                                             | 561–563           | SLK                  |
|                                             | 656–657 (130)     | SIR                  |
|                                             | 700–702 (120)     | STK                  |
|                                             | 746–748 (130)     | STDD                 |
|                                             | 69–72             | TYEE                 |
|                                             | 121–124           | TKED                 |
|                                             | 200–206           | STED                 |
|                                             | 449–452           | TYES                 |
|                                             | 455–458           | SSKK                 |
|                                             | 553–556           | TAVE                 |
|                                             | 610–613           | TDDE                 |
|                                             | 655–658           | SIRE                 |
|                                             | 663–666 (130)     | SEESD                |
|                                             | 730–733 (120)     | STDD                 |
|                                             | 776–779 (130)     | STDD                 |
|                                             | 7–12              | GSNPTE               |
| Casein kinase II                            | 93–98             | GTCPNS               |
|                                             | 197–202           | GQKPSL               |
|                                             | 269–274           | GLSNKF               |
|                                             | 312–317           | GTPMQQ               |
|                                             | 429–434           | GVMNPS               |
|                                             | 597–602           | GSQSGG               |
|                                             | 659–664 (120)     | GMSVGR               |
|                                             | 705–710 (130)     | GMSVGR               |
| Myristoylation sites                        | 199–214 (120 × 130) | KPSLSTEDSQEENTSK          |
|                                             | 596–634 (130)     | HGGSGSGGMPPPPTDDEIY   |
|                                             | 596–631 (120)     | DIGEEEEEEDDSGVSVDQEK   |
|                                             | 661–677 (130)     | KVSESDDNEGSSLQPQR     |
|                                             | 766–781 (130)     | KPGESLEVIQSTDOTK      |
|                                             | 720–735 (120)     | KPGESLEVIQSTDOTK      |

The FYB-130 cDNA, a novel isoform of FYB. Comparison of mouse FYB-130 sequence with mouse and human FYB-120. A, mouse FYB-130 shows a complete conservation with mouse FYB-120 except for a 46-amino acid insert at murine residue 627. The inset is located between two tyrosine-based motifs (YDGI and YDDV). FYB-130 retains clusters of type 1 SH2 recognition motif (Arg-X-Pro-X-X-Pro) or type II (Pro-X-X-Pro-X-Arg) motifs (single underline), multiple tyrosine residues including the YDGI and YDDV motifs (bold type, broken underline), two putative nuclear localization motifs K/R/R/R-X1-X2-K/R/K/R (heavy underline), and an SH3-like domain (thin lined box) as previously noted for FYB-120. The FYB-130 inset of 46 amino acids is highlighted with a thick box. B, the FYB-130 has potential phosphorylation sites for protein kinase C, cAMP, or cGMP kinase (bold underline), two potential sites for casein kinase II (shading), and the presence of a predicted secondary helix structure (b). Overlapping PEST sequences are located at the amino- and carboxyl-terminal end of the insert (bold boxes). C, regions of homology of the FYB-130 with the nuclear proteins RecA, HMG 1/2, and the regulator of chromosome condensation. The boxed regions represent identities, and the + indicates amino acid similarities.
p13; in 7 metaphase spreads both copies of chromosome 5 were labeled, and in 11 metaphase spreads signals were detected on one chromosome 5. These data provided an accurate localization of the human gene at chromosome 5p13.1 (designation FYB (alias SLAP) at 5p13.1 has been approved by the HUGO/GDB Nomenclature Committee).

**DISCUSSION**

Recent progress has been made in the identification of lymphoid-specific adaptors involved in the transmission of signals needed for IL-2 expression in T-cells (8, 9). FYB/SLAP is one such lymphoid protein with the hallmarks of an adaptor protein that binds to the SH2 domains of FYN-T and SLP-76 (23, 26). Binding to and phosphorylation by FYN-T provides a novel mechanism to implicate this kinase in signaling from the TcR. FYB also binds to the SH2 domain of SLP-76, a key protein in signaling from the pre- and mature TcR (13, 14). The original
FYB-130, Novel Isoform of FYB/SLAP

FIG. 4. Differential expression of FYB isoforms on thymocytes and mature T-cells. A, fluorescent-activated cell sorter analysis of total and depleted thymocytes. Thymocytes were isolated and subjected to immunodepletion as described under “Materials and Methods.” Upper left panel, Ig-fluorescent isothiocyanate (FITC) Phycoerythrin (PE) control; lower left panel, total thymocytes stained for anti-CD4 and -CD8; upper right panel, thymocytes depleted for anti-CD3 and stained for anti-CD4 and -CD8; lower right panel, thymocytes depleted for anti-CD4/CD8 and stained for anti-CD4 and -CD8. B, FYB-120 and -130 expression in total and depleted thymocytes. Cell lysates derived from cellular preparations enriched for CD4/CD8 double-negative thymocytes (lane 1), CD4/CD8 double-positive thymocytes (lane 2), total thymocyte populations (lane 3), a pro-thymocytic malignancy (lane 4), and splenic T-cells (lane 5) were separated by SDS/PAGE and subjected to blotting with anti-FYB. Densitometric analysis is shown in the lower panel and represents the relative amounts of FYB-120 and FYB-130 expressed as a percentage of the total FYB in each lane.

cDNA for FYB/SLAP encoded a protein that corresponded to the lower Mr form of the FYB doublet (termed FYB-120 or SLAP-130) (23, 24). In this study, we report the identification of a novel isoform of FYB/SLAP that differs from FYB-120 due to a unique insert of 46 amino acids that is located between two tyrosine-based motifs YDGI and YDDV as well as between the two putative nuclear localization motifs (Fig. 1A). This insert is enriched with a stretch of lysine and aspartic acid residues (KGDDDRKK) and carries putative phosphorylation sites for the serine/threonine kinases such as casein kinase II, protein kinase C, and cAMP or cGMP kinases. Since these sites are not present in the FYB-120 isoform, the insertion provides an alternative mode of regulation of FYB-130. At the same time, FYB-130 and -120 share a number of structural features. Kyte-Doolittle hydrophobicity analysis was performed to compare further the overall structural information (data not shown). FYB-120 and FYB-130 are surprisingly hydrophilic, with only six or eight small regions of relative hydrophobicity, respectively. FYB-130i adds hydrophobic and hydrophilic regions to the primary structure. The predicted helical structure in FYB-130i comprises mostly hydrophobic residues. Both FYB-120 and FYB-130 possess other regions of interest that have not been previously reported. These include an uncharged amino acid region at residues 364–409 that is enriched with proline (34.8%), serine (17.4%), threonine (10.9%), and alanine (10.9%), and mixed charged clusters of amino acids occurred in regions that include the putative nuclear localization motif at residues 440–491 and contains high proportions of lysine (25%) and glutamate (34.6%) (section B). Another area from residues 714 to 734 (FYB-130) and 668 to 683 (FYB-120) contained high proportions lysine (37.9%), glutamate (17.2%), and aspartate (10.3%) corresponding to the second nuclear localization motif. Negatively charged clusters of amino acids are also found between residues 611–630 (FYB-130), a region enriched for aspartate (35%) and glutamate (20%). This corresponds to the region at the amino-terminal of the 130i. An uncharged pocket was found at 626–629, adjacent to the amino-terminal of FYB-130i. FYB binding partner SLP-76 also has an unusually high overall hydrophilicity (data not shown).

FYB-120 and FYB-130 share the ability to bind to the FYN-T and SLP-76 proteins and, importantly, to act as positive regulators of TcR-mediated signals leading to IL-2 gene transcription in T-cells. FYB-130 was also found to act as a target of phosphorylation by the FYN-T kinase (Fig. 2). Both isoforms underwent equivalent levels of phosphorylation. This finding combined with the observation that FYB-120 and FYB-130 phosphorylation is reduced in T-cells from FYN-T−/− mice implicates both isoforms in a cascade involving the FYN-T kinase (25). We have recently shown that the YDGI motif binds to the FYN-T SH2 domain, whereas the YDDV site mediates SLP-76 SH2 binding (26). Therefore, although the insert is located between two tyrosine-based motifs, they are still accessible to SH2 recognition. A small but consistent difference between the two isoforms was noted where the FYN-T SH2 and SLP-76 SH2 domains precipitated greater amounts of FYB-120. The presence of the FYB-130i could therefore partially interfere with the accessibility of the binding sites for the FYN-T and SLP-76 SH2 domains.

In addition to FYN-T and SLP-76 binding, FYB-130 was found to localize in the cytoplasm and nucleus of cells (Fig. 3). FYB-120 had previously been found to localize in the cytoplasm, often showing perinuclear staining (26, 39). Here, we extend this observation by demonstrating that FYB-120 and FYB-130 can also be found in the nucleus of COS cells (Fig. 3). In the nucleus, both FYB 120 and FYB-130 showed a punctate or dotted pattern that was excluded from nucleoli. This dotted
pattern was even more striking using an anti-FYB serum to stain endogenous FYB in the cytoplasm and nucleus of T-cells (Fig. 3) and is suggestive of a high degree of protein compartmentalization. Given this observation and the fact that the FYB-130 insert shares homology with other nuclear proteins, it is possible that the insert plays a role in the nucleus. Consist-

Fig. 5. FYB-120 and FYB-130/FYN-T/SLP-76 augments TcR-mediated NFAT/AP-1 promoter activity. Upper panel, Jurkat T-cells were co-transfected with 5 µg of IL-2/NFAT/AP-1 luciferase reporter plasmid and 0.2 µg of RL-TK plasmid together with either 20 µg of empty vector, HA-FYB-120, HA-FYB-130, HA-FYB-120 or -130 with FYN-T, HA-FYB-120, or -130 with FYN-T and HA- SLP-76 and HA-FYB-120 and HA-FYB130 together with FYN-T and HA- SLP-76. Cells were either unstimulated (gray bars) or stimulated with either OKT3 (white bars) or OKT3 plus phorbol 12-myristate 13-acetate (10 ng/ml) (black 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 three independent experiments. Lower panel, cell lysates of the transfected Jurkat cells as described above were prepared and analyzed by immunoprecipitation and immunoblotting with anti-HA.

Fig. 6. Chromosome localization of the human FYB gene. A, idiogram of human chromosome 5. Idiogram showing the map localization of the human FYB gene at 5p13 (arrow). B, photograph of human metaphase chromosomes counterstained with DAPI. The two chromosomes 5 are indicated by numbers. Arrows point to the site of hybridization of the digoxigenin-labeled human FYB probe on both chromosomes 5 in band p13. Hybridization was observed with a Zeiss Axiophot microscope and images captured and printed using the Cytovision Imaging System (Applied Imaging, Pittsburgh, PA).
ent with the nuclear localization, FYB possesses several lysine/glutamic acid-rich clusters (29 of 40 residues) with similarity to bipartite nuclear localization motifs K/R/R-X_{1–12}-K/R/K (40). Overall, these observations suggest that FYB will be found to function in both the cytoplasm and nucleus of T-cells.

One difference between FYB-120 and FYB-130 was their relative expression in thymocytes and mature T-cells (Fig. 4). FYB-130 was more abundant relative to FYB-120 in mature T-cells, whereas thymocytes showed a greater relative amount of FYB-120. Thymic differentiation follows a pattern from immature CD+CD8−CD3− cells to CD4+CD8+CD3+ cells and finally more mature CD4+CD8+CD3+ or CD4+CD8−CD3+ single positive thymocytes (37). Enrichment of thymic subpopulations with immature thymocytes confirmed the skewing in favor of FYB-120 expression. Therefore, given this preferential expression in the thymus, it is possible that FYB-120 plays a more predominant role in thymic differentiation than FYB-130. In general, a role for FYB in thymic differentiation would be consistent with its binding to SLP-76, an adaptor recently shown to be required for pre-TcR signaling (13, 14).

Given its preferential expression on mature T-cells, FYB-130 may have become adapted to provide a more specialized function in these cells, possibly in a manner related to TcR-driven IL-2 production. FYB-130 was particularly potent in potentiating TcR-driven NF-AT/AP-1 transcriptional activity in stimulated Jurkat cells when co-expressed with FYN-T and SLP-76 (Fig. 5). FYB-130 was previously found to cause an up-regulation of TcR-driven IL-2 transcription in T-cells when co-expressed with FYN-T and SLP-76 (26). Under the same conditions, FYB-130 was generally found to provide more potent signal than FYB-120, a observation that is consistent with its higher levels of expression in mature T-cells. At the same time, unlike in the case of synergy with SLP-76 and FYN-T, co-expression of FYB-120 and FYB-130 failed to show synergy in the regulation of transcription. Consistent with this, we have failed to detect binding between the two isoforms in biochemical studies (data not shown). This suggests that each isoform is capable of interacting independently with SLP-76 and FYN-T in the generation of signals. Given their differential expression, they could compete with each other for engagement of the SLP-76 and FYN-T proteins in different cells. Further studies will be needed to determine the degree to which the FYB-130i is responsible for the enhanced functional effects of FYB-130 on transcription.

The generation of two isoforms suggests that they arise from differential splicing, an observation confirmed by preliminary Southern blot analysis (data not shown). Chromosomal mapping showed that FYB gene is located on chromosome 5 at position p13.1 (Fig. 6). This is the site of the glial cell-derived neurotrophic factor gene and has been linked to neurologic Hirschsprung disease (41). It is also the site of the interleukin 7 receptor and the complement factors C6, C7, and C9. IL-7 receptor signals support positive selection of early TcR-positive T-cells and in the development of pre-B-cells (42, 43). The growth factor gene is also located in the region, the site of forms of dwarfism (i.e., Laron syndrome, Idiopathic short stature) that are associated with T-cell immune deficiencies (44). It is presently unclear whether any deletions associated with dwarfism might involve a loss of FYB gene function or integrity. The FYB gene is also located on the same chromosome as the SLP-76 gene (5p33.1) (45). Further studies will be needed to investigate further the relative roles of FYB-120 and FYB-130 in T-cell function/differentiation and in immunodeficiencies.

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