The Src-Family Kinase, Fyn, Regulates the Activation of Phosphatidylinositol 3-Kinase in an Interleukin 2-responsive T Cell Line

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Summary

The proliferation of antigen-activated T cells is mediated by the T cell-derived growth factor, interleukin 2 (IL-2). The biochemical signaling cascades initiating IL-2-induced growth are dependent upon protein tyrosine kinase (PTK) activity. One IL-2-regulated PTK implicated in this cascade is the Src-family kinase, Fyn. Previous studies have described a physical association between Fyn and a potential downstream substrate, phosphatidylinositol 3-kinase (PI3-kinase) as well as the IL-2-dependent activation of PI3-kinase in T cells; however, the role of Fyn in IL-2-induced PI3-kinase activation remains unclear. In this report, we demonstrate that IL-2 stimulation triggers tyrosine phosphorylation of the p85 subunit of PI3-kinase in the murine T cell line, CTLL-2. Lysates prepared from growth factor-deprived and IL-2-stimulated T cells reconstituted both the binding of CTLL-2 cell-derived Fyn to and the IL-2-inducible tyrosine phosphorylation of exogenously added recombinant p85. Furthermore, overexpression of wild-type Fyn in these cells enhanced both the basal and IL-2-mediated activation of PI3-kinase. Additional studies of the Fyn-PI3-kinase interaction demonstrated that the Src homology 3 (SH3) domain of Fyn constitutes a direct binding site for the p85 subunit of PI3-kinase. These results support the notion that Fyn may be directly involved in the activation of the downstream signaling enzyme, PI3-kinase, in IL-2-stimulated T cells.

Antigenic stimulation of resting T cells initiates entry into the cell cycle (G_0- to G_1-phase transition) and the synthesis of both the IL-2 receptor (IL-2R)[1] and its cognate ligand, IL-2. IL-2R occupancy then triggers G_1- to S-phase progression, and commits the cell to traverse the remainder of the cell cycle. Although the biological effects of IL-2 on immune cell function are well known, the intracellular biochemical mechanisms coupling IL-2R stimulation to IL-2-induced T cell mitogenesis are poorly understood.

The high-affinity IL-2R is minimally a heterotrimeric complex consisting of 55-kD (α, p55), 70-kD (β, p70), and 64-kD (γ, p64) polypeptide subunits (reviewed in 1). Although all three subunits are required for high-affinity binding to ligand, the human p70 and p64 subunits comprise an intermediate affinity receptor, and the p70-p64 heterodimer can confer mitogenic responsiveness to IL-2 (2, 3). The IL-2R p70 and p64 subunits are members of the cytokine receptor superfamily. Like other members of this family, the deduced amino acid sequences of the cytoplasmic domains of the IL-2R subunits predict no homology to protein kinase catalytic motifs, yet IL-2R occupancy elicits a rapid increase in the tyrosine phosphorylation of multiple intracellular proteins (4-6). The Src-family kinases, Lyn, Fyn, and Lck have been postulated to function as IL-2R-linked protein tyrosine kinase (PTK). All three are rapidly and transiently activated by IL-2R stimulation (7, 8). Moreover, both Fyn and Lck are physically associated with the IL-2R complex (8-10). These findings suggest that certain Src-family PTKs may serve wholly or partially redundant functions in the transmission of the IL-2R-initiated mitogenic signal (8-12). However, the molecular mechanisms that target these receptor-activated PTKs to downstream signal-transducing molecules remain enigmatic.

Previous studies have shown that IL-2 stimulation of T cells results in the rapid PTK-dependent activation of phosphatidylinositol 3-kinase (PI3-kinase) (13-15). In addition, PI3-kinase is coprecipitated with the IL-2R complex (15) and the Src-family kinase, Fyn (13). While the actual function of PI3-kinase is unknown, the enzyme appears to be a ubiquitous and essential component of PTK-initiated mitogenic response pathways (16). PI3-kinase is a heterodimer composed of an 85-kD regulatory subunit and a 110-kD catalytic subunit (17, 18). Two potential molecular mechanisms for the cata-

[1] *Abbreviations used in this paper:* GSH, glutathione; GST, glutathione S-transferase; IL-2R, IL-2 receptor; PI3-kinase, phosphatidylinositol 3-kinase; PTK, protein tyrosine kinase; Ptyr, phosphotyrosine; SH, Src homology.

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lytic activation of PI3-kinase have been identified (19). According to one model, the Src homology 2 (SH2) domains of p85 bind to autophosphorylated growth factor receptors or their substrates. This interaction induces an allosteric alteration that activates the 110-kD catalytic subunit. The alternative model predicts that tyrosine phosphorylation of p85 itself increases the catalytic activity of the 110-kD subunit. However, the PI3-kinase activation mechanism employed by the IL-2R is currently unknown.

To more fully dissect the biochemical linkages among the IL-2R, the Src-family kinases, and downstream signal-transducing moieties, we have focused on the IL-2–induced activation of PI3-kinase. The studies described herein demonstrate that the p85 subunit of PI3-kinase is a direct target for an IL-2–inducible tyrosine kinase in intact T cells. In vitro studies demonstrated the binding of T cell–derived Fyn to a recombinant p85 fusion protein and the IL-2–inducible tyrosine phosphorylation of the recombinant p85. In addition, overexpression of wild-type Fyn in CTLL-2 cells enhanced both basal and IL-2–stimulated PI3-kinase activity. Using recombinant fusion proteins, we observed that the isolated Fyn SH3 domain could bind directly to the p85 subunit of PI3-kinase. Taken together, these results define a direct coupling mechanism between IL-2R–mediated Fyn activation and the subsequent phosphorylation and activation of the downstream signaling enzyme, PI3-kinase.

Materials and Methods

Reagents. The anti-p85 rabbit antiserum and the antiphosphotyrosine (Ptyr) mAb, 4G10, were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The rabbit anti-Fyn antiserum has been described previously (11). The IL-2 was a generous gift of Hoffmann-LaRoche, Inc. (Nutley, NJ). The anti-KT3 mAb (20) was obtained from G. Walter (University of California, San Diego, CA). The pGEX-KG vector (21) was from J. Dixon (University of Michigan, Ann Arbor, MI), the pREP3 vector was from J. Tykocinski (Case Western Reserve University, Cleveland, OH [22]), and the murine p85 cDNA (17) was obtained from L. Williams (University of California, San Francisco, CA). The *fyn* and *lck* cDNAs were generous gifts of R. Perlmutter (University of Washington, Seattle, WA).

Plasmid Construction. To construct glutathione S-transferase (GST)-Fyn^{NH} (amino acids 1–85) and GST-Fyn^{SH3} (amino acids 84–141) expression vectors, the respective oligonucleotide pairs 5′-CGACAGGATCCGCTAGATACCATGGGCTGTGTGCAATG-3′, 5′-CGAGCAGCTGAAGCTTTGTCACTCCTGTCCCTCC-3′, and 5′-CGACAGGATCCTCTAGATACCATGGTGACACTGTTTGTGGCG-3′, 5′-CGAGCAGCTGAAGCTTAACTGGAGCCACGTAAT-3′ were used to prime PCRs. The amplification products were digested with HindIII and XbaI and cloned into the pGEX-KG vector backbone digested with the same restriction enzymes. All PCR-derived clones were sequenced to verify that no errors were introduced. To construct the GST-FYN$^{NH-SH3-SH2}$ (amino acids 25–265). The *fyn*-containing vector, pMTFR, was digested with SacI, and the resulting *fyn* fragment (encoding amino acids 25–358) was ligated into SacI-digested pSP72 (Promega Corp., Madison, WI). The pSP72-Fyn construct was digested with EcoRV and XbaI and ligated into the SmaI- and XbaI-digested pGEX-KG vector. The fusion protein produced from this vector was insoluble. To truncate the fusion protein, the plasmid was digested with PMlI (truncating the protein at amino acid 265) and HindIII (cutting in the pGEX-KG polylinker 3′ to the insert). The DNA ends were rendered flush by treatment with the Klenow fragment of *Escherichia coli* DNA polymerase, and the fragments religated under dilute conditions. The resulting plasmid was designated pGEX-KG-FYN$^{NH-SH3-SH2}$ (amino acids 25–265). To construct the GST-Fyn^{SH2} expression plasmid, a HincII to PmlI fragment of *fyn* (amino acids 142–265) was excised from pMTRF and subcloned into EcoRV-digested pSP72. pSP72-Fyn^{SH2} was then digested with BglII and EcoRI and the *fyn*-containing fragment was ligated into BamHI- and EcoRI-digested pGEX-KG.

To generate the GST-Lck fusion protein expression vector, the *lck*-containing vector, pMNC-LCK, was first digested with PstI to release an *lck* fragment encoding amino acids 5–295. The DNA fragment was ligated into PstI-digested pGEM3-Zf(−). To generate an inframe fusion with the GST coding sequences, the pGEM-Lck vector was digested with XbaI and HindIII and the resulting *lck* fragment was subcloned into XbaI- and HindIII-digested pGEX-KG. The fusion protein expressed from this vector was insoluble. Therefore, the vector was digested with NcoI (cuts within the *lck* coding sequence at amino acid 232) and HindIII (cuts within the polylinker sequence, 3′ to the *lck* insertion). The vector-containing fragment was treated with the Klenow fragment of *E. coli* DNA polymerase, and the plasmid was religated under dilute conditions.

To generate the GST-p85 expression plasmids, the vector containing the p85 cDNA was digested with PstI. To construct GST-p85$^{\Delta A158-724}$, the p85 plasmid was digested to completion with PstI and the resulting fragment (encoding amino acids 158–724 of p85) was subcloned into pSP72. The resulting plasmid was digested with SalI and HindIII and the p85-containing fragment was ligated into SalI- and HindIII-digested pGEX-KG. To prepare the GST-p85$^{\Delta A1-724}$ expression vector, the p85 plasmid was partially digested with PstI. The fragment encoding full-length p85 was gel-purified and ligated into PstI-digested pSP72. This plasmid was digested with SalI and HindIII, and the p85-containing fragment was ligated into SalI- and HindIII-digested pGEX-KG. This insertion generated an inframe insertion with the GST coding sequence.

To construct the epitope-tagged Fyn expression vector, pREP3-Fyn^{wt}-KT3, the 8-amino acid KT3 epitope tag (TPPEPET) was appended onto the 3′ end of the full-length *fyn* coding sequence using the polymerase chain reaction. The oligonucleotides, 5′-GAATTCGATATCTAGAAATGGGCTGTCTGCAATGT-3′ and 5′CGATGCATGCCTCGAGTCATGTTTCTGGTTCTGGTGGTGGTGTCAGGTTTTCACCGGGCTG-3′ were used to prime the polymerase chain reaction. The resulting amplification product was digested with XhoI and XbaI and the fragment was ligated into NheI- and XhoI-digested pREP3.

Preparation of Fusion Proteins. Fusion proteins were expressed in the *E. coli* strain DH5α transformed with the appropriate vectors. The cultures were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside and incubated for 2–3 h for production of GST-Fyn fusion proteins and 20 min for the preparation of GST-p85 fusion proteins. The cells were resuspended in 0.01 M sodium phosphate, 0.15 M NaCl, 10 mM EDTA, (pH 7.4) 10 mM dithiothreitol, 1% Triton X-100, and 0.03% SDS containing 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 1 mM PMSF, and disrupted by sonication. The GST fusion proteins were purified using glutathione (GSH)-coupled agarose (Sigma Chemical Co., St. Louis, MO).

Cell Culture. The CTLL-2 cells were obtained from American Type Culture Collection (Rockville, MD). The cell culture conditions, as well as the derivation of the CTLL.lck$^-$ cell line, were described previously (11). Where indicated, CTLL-2 cells were

starved of growth factors for 10–12 h before experimental manipulations by culturing in RPMI 1640 medium supplemented with 1% BSA, 2 mM L-glutamine, 50 μM 2-ME, and 10 mM Hepes (pH 7.2).

Cell Transfections. Exponentially growing CTLL-2 cells (10^7 cells/transfection) were mixed with DNA. The mixture was incubated at room temperature for 10 min and electroporated at 475 V, 600 μF in a 0.4-mM cuvette using an ECM 600 apparatus (BTX, Inc., San Diego, CA). After electroporation, the cells were incubated at room temperature for 15 min and diluted into culture medium. After 24 h, 400 μg/ml hygromycin B was added. Drug-resistant cells were cloned by limiting dilution. To identify cells lines expressing epitope-tagged Fyn, the tagged Fyn was immunoprecipitated with the KT3 monoclonal antibody (20), and the precipitates were subjected to a kinase reaction as described below. The Fyn-KT3–expressing clones were identified by the presence of ^{32}P-labeled autophosphorylated Fyn.

Immune Complex and Fusion–Protein Kinase Reactions. Immune complexes or fusion protein precipitates were washed with immune complex kinase buffer (50 mM Pipes [pH 7.0], 0.02% Triton X-100, 10 mM NaCl, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 1 mM Na_3VO_4). The immune complexes were subjected to kinase reactions by addition of 10 or 20 μCi γ-[^{32}P]ATP (50 Ci/mmol) and, where indicated, 5 μg acid-denatured enolase (11). Proteins were fractionated by SDS-PAGE, and transferred to Immobilon P as previously described (11). Radiolabeled proteins were visualized by autoradiography, and protein-bound radioactivity was quantitated with an imaging system (AMBIS Systems Inc., San Diego, CA).

Phosphorylation of p85 in Intact Cells. IL-2–deprived CTLL-2 cells (2×10^7 cells/sample) were washed once with PBS (0.01 M sodium phosphate, 0.15 M NaCl [pH 7.4]) containing 1 mg/ml glucose and resuspended in 1 ml of the same buffer. After warming to 37°C, the cells were either pretreated or not for 2 min with 30 μM vanadyl hydroperoxide (pervanadate; [23, 24]). Samples were then stimulated with medium or IL-2, and the cells were lysed in PI3-kinase lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 40 mM β-glycerophosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol [pH 7.4]) containing 1% Triton X-100, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 1 mM PMSF. Cell lysates were immunoprecipitated anti-p85 antiserum. The immune complexes were washed with PI3-kinase lysis buffer containing 1.0% Triton X-100, 1.0% deoxycholate, 0.1% SDS, and 1 mM Na_3VO_4, and were fractionated by SDS-PAGE. The proteins were transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA), and the membrane was probed with an anti-Ptyr mAb as previously described (11). The membrane was then stripped with 7 M guanidine hydrochloride, 10 mM dithiothreitol, and immunoblotted with anti-p85 antiserum, followed by a 1:10,000 dilution of a horseradish peroxidase–protein A conjugate (Amersham Corp., Arington Heights, IL). Immune complexes were detected with the enhanced chemiluminescent detection reagent (ECL; Amersham Corp.).

Fyn-p85 Binding Assays. Purified GST-Fyn fusion proteins (in 0.2 M sodium bicarbonate) were coupled to cyanogen bromide-activated Sepharose (Sigma Chemical Co.) at a concentration of 0.2 μmol of fusion protein/ml of resin. For each binding reaction, 15 μl of packed beads were mixed with 20 μg of full-length, soluble GST-p85$^{AA1-724}$ (amino acids 1–724) in 1 ml of PI3-kinase lysis buffer containing 1 mM dithiothreitol, 0.5% Brij 96, 10 mg/ml BSA, and 2 μg/ml of soluble GST. The soluble GST served as a competitor for a weak background GST-GST-p85 interaction. The samples were rotated for 1 h at 4°C, washed with lysis buffer, and fractionated by SDS-PAGE (10% gel). The gel was transferred to Immobilon P and the membrane was immunoblotted with anti-p85 antiserum, with the exception that the blotting solution was precleared of GST-reactive antibodies by tandem 2-h incubations with 500 μg of GST bound to GSH-Sepharose, followed by a single incubation with GSH-Sepharose only. Finally, 500 μg of SDS-denatured GST (prepared by heating for 10 min at 100°C in 0.1% SDS) were added to the blotting solution.

GST-p85 Binding and Kinase Assays. Growth factor–deprived CTLL-2 cells were stimulated for the indicated times with 200 U/ml IL-2. The cells were lysed in PI3-kinase lysis buffer containing 0.25% Triton X-100, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 1 mM PMSF. Cell lysates were incubated with 10 μg GSH–agarose-bound GST-p85 $^{AA158-724}$ for 1 h, and the resulting precipitates were subjected to kinase reactions as described above. Phosphoamino acid analyses were performed on the ^{32}P-labeled, membrane-bound GST-p85 bands as previously described (25).

A parallel set of GST-p85–based kinase reactions was analyzed for radiolabeled Fyn. After the kinase reaction, the samples were adjusted to 1% SDS and heated for 20 min at 100°C. After heating, the samples were diluted with PI3-kinase lysis buffer containing 1% Triton X-100 to a final SDS concentration of 0.1%. Insoluble material was removed by centrifugation, and the supernatant was immunoprecipitated with anti-Fyn antiserum. The immunoprecipitates were fractionated by SDS-PAGE. The gel proteins were transferred to Immobilon P and the membrane was exposed to film.

PI3 Kinase Assays. Wild-type CTLL-2 or lck-deficient CTLL-2 (CTLL.lck$^-$) cells were deprived of IL-2 for 12 h and then restimulated for 15 min with 200 U/ml recombinant IL-2. At the end of the reaction, the cells were lysed in PI3-kinase lysis buffer containing 0.25% Triton X-100, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin, and 1 mM PMSF. Cleared lysates were incubated with either anti-Ptyr mAb–coupled Sepharose (4), GSH-agarose–bound GST-Fyn fusion protein, or GST-Fyn–coupled Sepharose. The precipitates were washed with PI3-kinase lysis buffer containing 0.1% Triton X-100, 1 mM Na_3VO_4, and 0.2 mM adenosine, and with 10 mM Tris-HCl, 100 mM NaCl (pH 7.6) containing 1 mM Na_3VO_4, and 0.2 mM adenosine. PI3-kinase reactions were performed as previously described (13).

Results

IL-2 Induces the Tyrosine Phosphorylation of p85. Stimulation of T cells with IL-2 results in an increase in PI3-kinase activity recoverable in anti-Ptyr mAb immunoprecipitates (13–15). To determine if this increase is due, in part, to the tyrosine phosphorylation of a component of PI3-kinase itself, we stimulated IL-2–deprived CTLL-2 cells with a saturating concentration of IL-2 in the presence or absence of pervanadate, a potent tyrosine phosphatase inhibitor in intact T cells (23, 24). Subsequently, detergent-soluble proteins were immunoprecipitated with anti-p85 antiserum, and the immunoprecipitates were resolved by SDS-PAGE and immunoblotted with an anti-Ptyr mAb. Stimulation of nonpretreated CTLL-2 cells with IL-2 for 15 min elicited a readily detectable increase in the tyrosine phosphorylation of p85 (Fig. 1 *A*). In pervanadate-pretreated cells, p85 exhibited a basal level of tyrosine phosphorylation that was comparable to that induced by IL-2 in nonpretreated cells (Fig. 1 *A*, compare lanes *2* and *3*). Stimulation of the pervanadate-pretreated cells with IL-2 resulted in a marked increase in the tyrosine phosphory-

lation of p85. The IL-2–induced tyrosine phosphorylation of p85 has been reproducibly observed in at least three separate experiments. Fig. 1 *C* demonstrates that neither IL-2 nor pervanadate treatment affected the recovery of p85 in the immunoprecipitates.

To determine whether enhanced levels of p85 phosphorylation were correlated with increased PI3-kinase activity, CTLL-2 cells were treated with pervanadate and IL-2 as described for Fig. 1 *A*, and anti-Ptyr antibody-bound PI3-kinase activities were determined (Fig. 1 *B*). The results indicate that increased levels of p85 phosphorylation were accompanied by the increased recovery of PI3-kinase activities in the anti-Ptyr mAb precipitates. Although pervanadate increased the basal tyrosine phosphorylation of p85 (as determined by anti-Ptyr blotting) to levels similar to that observed with IL-2 alone, it dramatically enhanced the PI3-kinase activity bound to anti-Ptyr immunoprecipitates. This discrepancy may reflect the enhanced tyrosine phosphorylation of the p110 subunit (Fig. 1 *A*), other posttranslational modifications of the p85–p110 heterodimer, or other protein–protein associations induced by the pervanadate treatment.

GST-p85 Binds an IL-2–inducible Tyrosine Kinase Activity. To examine the molecular machinery that couples the IL-2R to PI3-kinase, we attempted to recapitulate the IL-2–induced phosphorylation of p85 in vitro. Growth factor–deprived CTLL-2 cells were restimulated for various times with IL-2, and cell lysates were incubated with a GST fusion protein containing amino acids 158–724 of p85 (GST-p85$^{AA158-724}$) bound to GSH-coupled agarose beads. The washed precipitates were incubated with γ-[^{32}P]ATP in kinase buffer and were resolved by SDS-PAGE. IL-2 stimulation induced a rapid increase in protein kinase activity precipitated by the GST-p85$^{AA158-724}$ substrate (Fig. 2 *A*, *right*). In contrast, GST alone (Fig. 2 *A*, *left*) precipitated <5% of the kinase activity precipitated by GST-p85$^{AA158-724}$. In addition, this very low-level kinase activity was not IL-2 inducible. Repeat experiments indicated that IL-2 stimulated a two- to fourfold increase in GST-p85$^{AA158-724}$ phosphorylation. To determine whether the protein kinase activity phosphorylated GST-p85$^{AA158-724}$ on tyrosine or serine/threonine residues, phosphoamino acid analysis was performed on the GST-p85$^{AA158-724}$ band. The majority of the IL-2–inducible phosphorylation occurred on tyrosine residues, although a low basal level of serine phosphorylation was also evident (Fig. 2 *B*).

GST-p85 Binds IL-2–activated Fyn. To identify the PTK associated with the GST-p85 fusion protein, the kinase-bound GST-p85$^{AA158-724}$ precipitates were incubated with γ-[^{32}P]ATP, the protein complexes were disrupted with SDS, and the denatured proteins were reimmunoprecipitated with a Fyn-specific rabbit antiserum. The results in Fig. 2 *C* demonstrated that GST alone did not precipitate Fyn kinase activity. However, the GST-p85 precipitates contained ^{32}P-labeled Fyn. Additionally, Lck, the only other Src-family kinase expressed in these cells (11), could not be shown to specifically bind to the GST-p85$^{AA158-724}$ fusion protein.

If Fyn were responsible for the IL-2–induced phosphorylation of GST-p85, then IL-2–induced Fyn activation should coincide with the IL-2–dependent tyrosine phosphorylation of p85. To determine the time course of Fyn activation in response to IL-2, epitope-tagged Fyn (Fyn-KT3) was expressed in CTLL-2 cells. The addition of the KT3 epitope permitted selective recovery of the transfected Fyn with the KT3 mAb (20). Relative to immunoprecipitates prepared with our polyclonal rabbit anti-Fyn antisera, the KT3 immunoprecipitates contained significantly lower levels of nonspecific contaminating proteins, including immunoglobulin heavy chains (our unpublished observations). Growth factor–deprived, Fyn-KT3–expressing cells were restimulated with IL-2 for the indicated times. Cell lysates were precipitated with the KT3 mAb, and the precipitates were incubated in kinase buffer containing γ-[^{32}P]ATP (Fig. 3). Fyn activation was detected within 5 min of IL-2 stimulation, which was consistent with the rapid kinetics of p85 phosphorylation observed in the in vitro kinase assay. The KT3 mAb precipitated very low levels of nonspecific enolase-phosphorylating activity from either untransfected or empty vector-transfected CTLL-2 cells (data not shown). Collectively, these results demonstrate that

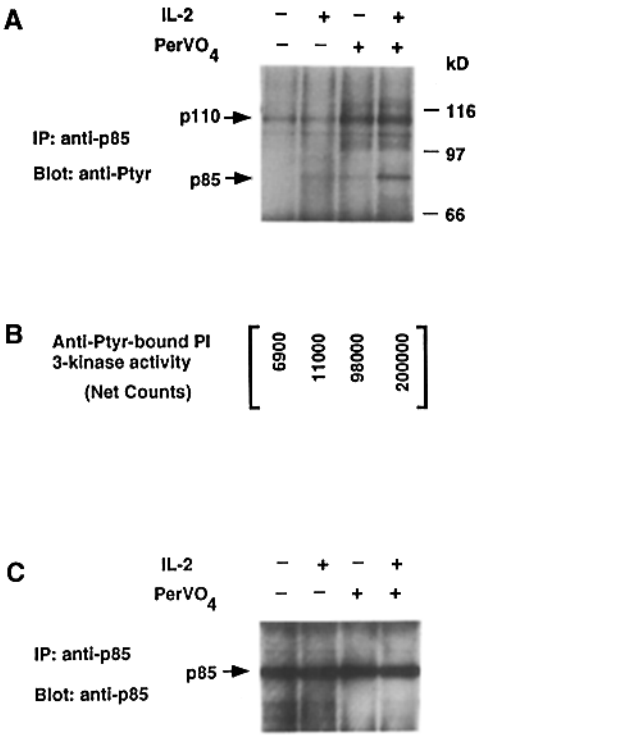

Figure 1. IL-2–induced tyrosine phosphorylation of p85 in intact cells. Two parallel sets of IL-2–deprived CTLL-2 cells were either pretreated or not with 30 μM pervanadate ($PerVO_4$) for 2 min at 37°C, and the cells were then stimulated for 15 min with medium only or with IL-2. (*A*) The anti-p85 antibody immunoprecipitates were fractionated by SDS-PAGE (10% gel). The gel proteins were transferred to Immobilon P, and the membrane was immunoblotted with an anti-Ptyr mAb. (*B*) Lysates from the second set of cells (stimulated as described in *A*) were precipitated with an anti-Ptyr mAb, and bound PI3-kinase activities were assayed. (*C*) The membrane shown in *A* was stripped and reprobed with an anti-p85 antiserum. The experiment shown is representative of three independent trials.

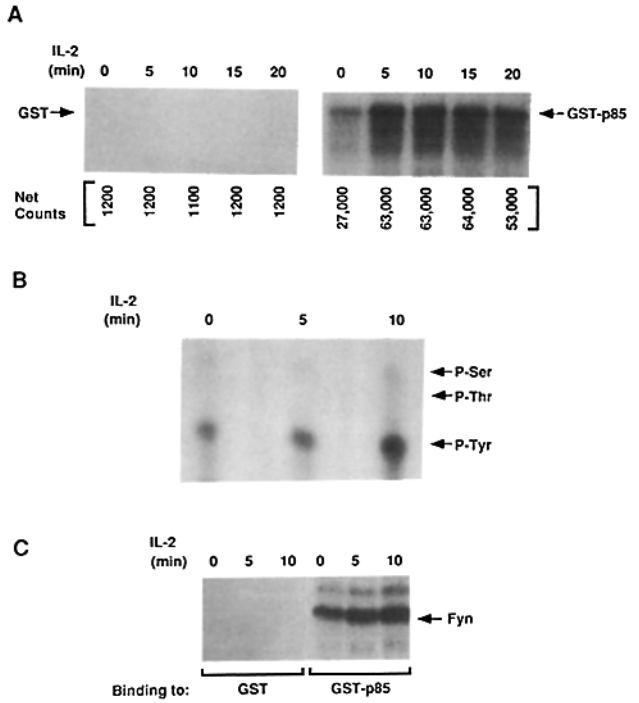

Figure 2. Association of an IL-2–activated PTK with recombinant p85. (*A*) Growth factor–deprived CTLL-2 cells were stimulated with IL-2 for the indicated times and lysed. Detergent-soluble proteins were concomitantly incubated with either GSH-agarose–bound GST (*left*) or GST-p85$^{AA158-724}$ (*right*), and the precipitates were incubated with γ-[^{32}P]ATP as described in Materials and Methods. Precipitated proteins were fractionated by SDS-PAGE (10% gel), transferred to Immobilon P, and radiolabeled species were detected by autoradiography. The incorporated radioactivity was quantitated with an imaging system (AMBIS Systems, Inc.). Data shown below *A* represent the number of counts detected in a 22-h data acquisition period (indicated as *Net counts*). Both phosphorylated GST and GST-p85$^{AA158-724}$ were autoradiographed for equivalent time periods. The low level of GST phosphorylation could not be reproduced photographically. (*B*) Phosphoamino acid analysis of the in vitro phosphorylated p85 band. The migration of unlabeled phosphoamino acid standards, added to each acid hydrolysate, is indicated. (*C*) Samples were prepared as in *A*, and after the in vitro kinase reactions, the protein complexes were disrupted with 1.0% SDS at 100°C. The samples were diluted to a final concentration of 0.1% SDS, and Fyn was reprecipitated with anti-Fyn antiserum and protein A-Sepharose. The autophosphorylated, ^{32}P-labeled Fyn band is indicated.

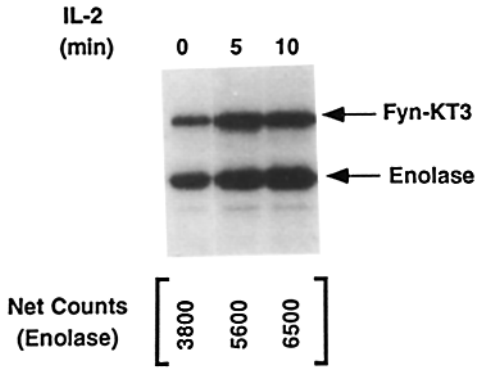

Figure 3. IL-2–induced activation of Fyn. IL-2–deprived, Fyn-KT3–expressing CTLL-2 cells were restimulated with IL-2 for the indicated times. Cell lysates were immunoprecipitated with the KT3 mAb, and the immune complexes were incubated with γ-[^{32}P]ATP. Acid-denatured enolase was added as an exogenous substrate. The reactions were terminated with Laemmli sample buffer and fractionated by SDS-PAGE (10% gel). Proteins were transferred to Immobilon P and radiolabeled proteins were detected by autoradiography. Quantitation of enolase *trans*-phosphorylation is indicated below the panel. Data represent the net counts detected in a 30-min data acquisition period. The migration positions of both Fyn and enolase are indicated.

recombinant GST-p85$^{AA158-724}$, like endogenous p85, complexes with T cell–derived Fyn, and undergoes IL-2–dependent tyrosine phosphorylation.

Fyn Enhances PI3-kinase Activation. The observations described above prompted us to examine the effect of overexpression of wild-type Fyn on both basal and IL-2–inducible PI3-kinase activities in CTLL-2 cells. Because Fyn and Lck may subserve overlapping functions in the regulation of PI3-kinase in IL-2–stimulated T cells (11, 26), we used a previously described variant (CTLL.lck$^-$) of the CTLL-2 cell line lacking Lck (11). These cells were transfected with an epitope-tagged Fyn expression vector and cloned by limiting dilution. Four Fyn-KT3–expressing cell lines were identified by screening for tyrosine kinase activity precipitable with the KT3 mAb. As controls, four cell lines that did not express Fyn-KT3 were used. These included the parental CTLL.lck$^-$ cell line, a bulk population of CTLL.lck$^-$ cells transfected with empty vector, and two clones (transfected with the Fyn-KT3 expression vector) that did not express any detectable Fyn-KT3 kinase activity. To examine the overall level of Fyn expression in these cell lines, total Fyn autophosphorylating activity was assessed in anti-Fyn immunoprecipitates (Fig. 4 *A*). The clones 9, 11, 17, and 23 all exhibited elevated levels of total Fyn kinase activity. As can be seen, the epitope-tagged Fyn displayed a slightly retarded migration in the polyacrylamide gel due to the addition of the peptide tag.

CTLL-2 cell subclones expressing normal or elevated levels of wild-type Fyn were subsequently assayed for anti-Ptyr–precipitable PI3-kinase activity. The cells were deprived of growth factor for 12 h, and equivalent amounts of detergent-soluble protein were immunoprecipitated with an anti-Ptyr mAb. As shown in Fig. 4, *B* and *C*, the cell lines overexpressing wild-type Fyn consistently displayed elevated basal and IL-2–inducible levels of PI3-kinase activities. Unexpectedly, the most dramatic alteration induced by wild-type Fyn overexpression was the 2.5- to 3-fold increase in immunoprecipitable PI3-kinase activities observed in IL-2–deprived CTLL-2 cells. This effect appeared specific for Fyn, as overexpression of either wild-type Lck (our unpublished data, 26) or Src (our unpublished data) failed to increase the level of anti-pTyr–precipitable PI3-kinase activities.

The SH3 Domain of Fyn Complexes with T Cell–derived PI3-kinase. Previous studies suggested a physical association between Fyn and PI3-kinase in IL-2–responsive T cells (13). Therefore, we analyzed the capabilities of isolated Fyn domains to mediate this interaction in vitro. GST-fusion pro-

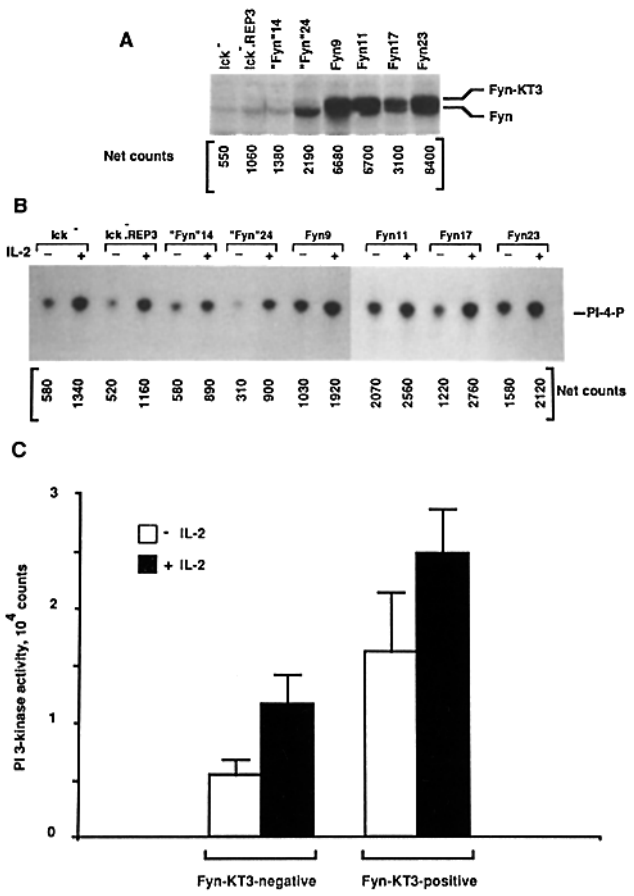

Figure 4. PI3-kinase activities in CTLL-2 cells expressing wild-type Fyn. (*A*) Analysis of epitope-tagged Fyn-KT3 expressing in transfected cells. Lysates from the indicated exponentially growing cell lines were immunoprecipitated with an anti-Fyn antiserum, and the immunoprecipitates were subjected to immune complex kinase reactions. The migration positions of epitope-tagged and endogenous Fyn are indicated. Total radioactivity in both bands was quantitated during a 30-min data acquisition period. The increased level of endogenous Fyn activity evident in the "Fyn"24 clone was not observed in repeat experiments. (*B*) IL-2-deprived CTLL-2 cells either not expressing (lck⁻, lck⁻.REP3, "Fyn"14, and "Fyn"24) or expressing Fyn-KT3 (Fyn.9, Fyn.11, Fyn.17, and Fyn.23) were stimulated for 15 min with IL-2. Cell lysates were precipitated with an anti-Ptyr mAb, and precipitated PI3-kinase activities were determined. The migration of the phosphatidylinositol-4-phosphate standard is indicated. Radioactivity in each spot was quantitated (see below *B*, labeled Net counts) and the data shown are net counts detected in a 180-min detection period. (*C*) Graphical summary of the data presented in Panel B. The means (± SD) of the four data points from the Fyn-KT3–negative cells and the Fyn-KT3–positive cells are shown. A Student's *t* test analysis of the data demonstrated a statistically significant enhancement of PI3-kinase activities for Fyn-KT3–positive cells compared with Fyn-KT3–negative cells in both IL-2–deprived ($p < 0.05$) and IL-2–stimulated ($p < 0.01$) samples.

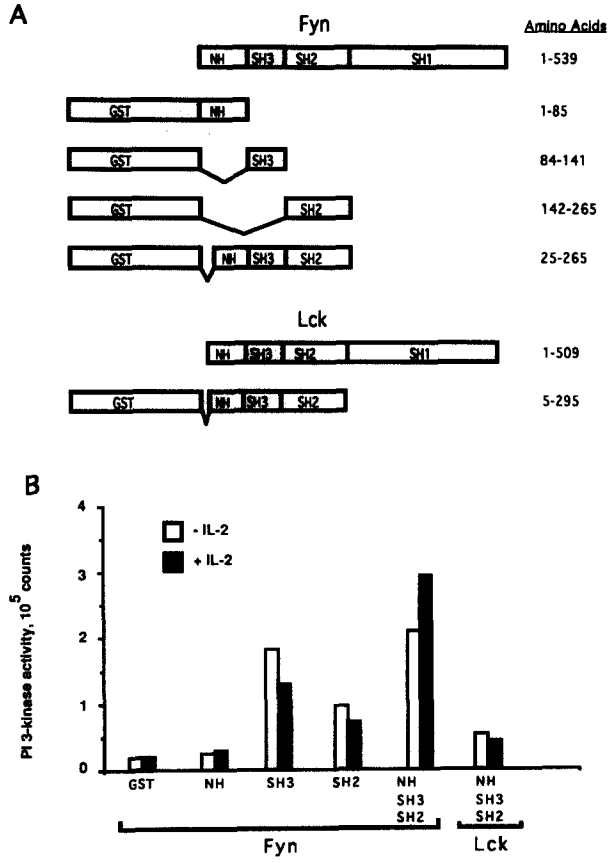

Figure 5. PI3-kinase activities binding to Src-family–derived fusion proteins. (*A*) Schematic diagram of GST-Fyn and GST-Lck fusion proteins used for the study. (*B*) IL-2–deprived CTLL-2 cells were stimulated or not with IL-2, and cell lysates were incubated with the indicated fusion proteins bound to GSH agarose. Fusion protein-bound PI3-kinase activities were assayed as described in Materials and Methods. Qualitatively similar results have been observed in three separate experiments.

teins were expressed in *E. coli* from pGEX vectors containing inframe insertions of the coding sequences for either the NH_2 terminus through the SH2 domains (amino acids 25–265), NH_2 terminus alone (amino acids 1–85), SH3 domain alone (amino acids 84–141), or SH2 domain alone (amino acids 142–265) of Fyn (Fig. 5 *A*). In addition, the coding sequences for the NH_2 terminus through the SH3 domain of Lck (amino acids 5–295) were also inserted into the pGEX vector. To assess the binding of endogenous PI3-kinase to the purified GST-fusion proteins, CTLL-2 cells were deprived of growth factor and either restimulated with medium alone or IL-2. Cell lysates were incubated with GSH-agarose–immobilized fusion proteins, and the resulting precipitates were assayed for PI3-kinase activities (Fig. 5 *B*). A low level of PI3-kinase activity was consistently precipitated by GST alone. The NH_2 terminus of Fyn did not bind PI3-kinase activity above the GST background. In contrast, both the isolated Fyn SH3 and SH2 domains complexed significant levels of PI3-kinase activity from CTLL-2 cell lysates, and a fusion protein containing both the Fyn SH2 and SH3 domains reproducibly bound the highest levels of PI3-kinase activity in these experiments. A fusion protein containing the NH_2 terminus, the SH3, and SH2 domains of Lck complexed much less CTLL-2 cell–derived PI3-kinase activity than did the analogous Fyn GST-fusion protein, suggesting that active PI3-kinase preferentially interacts with Fyn in this system.

The SH3 Domain of Fyn Binds Directly to the p85 Subunit

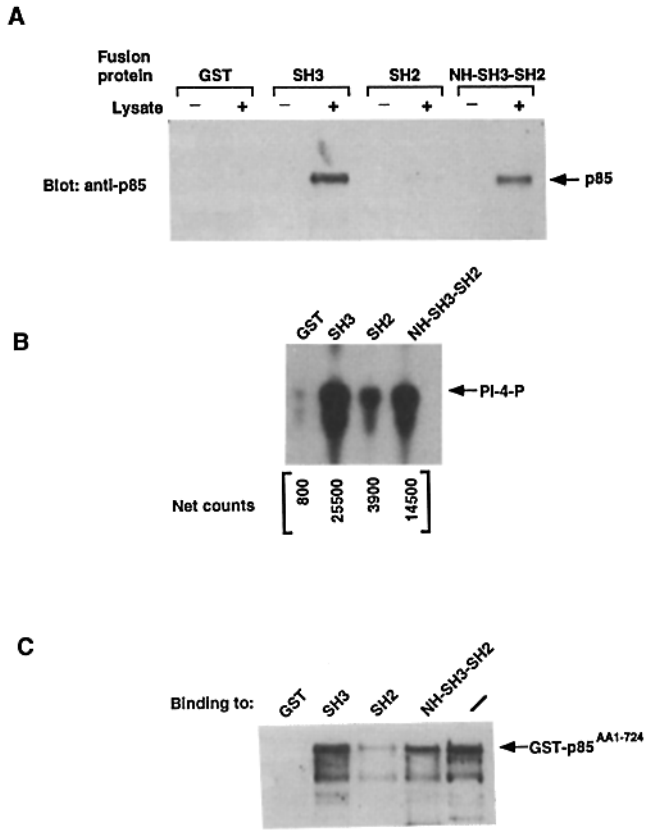

Figure 6. The p85 subunit of PI3-kinase binds directly to the SH3 domain of Fyn. (*A*) GST- or GST-Fyn fusion protein-coupled Sepharose beads were incubated with lysis buffer only (−), or with lysates prepared from exponentially-growing CTLL-2 cells (+). The protein complexes were washed and fractionated by SDS-PAGE (10% gel). The gel proteins were transfered to Immobilon P and probed with an anti-GST-p85 antiserum that had been depleted of GST-reactive antibodies. The cell lysate-derived, anti-p85–immunoreactive band is indicated. (*B*) Parallel binding reactions were assayed for GST fusion protein-bound PI3-kinase activities. The migration of the phosphatidylinositol-4-phosphate standard is indicated. The data below *B* indicate net counts detected during a 60-min data acquisition period. (*C*) The GST- and GST-Fyn-coupled Sepharose beads were incubated with GST-p85$^{AA1-724}$. The precipitates were fractionated by SDS-PAGE, and immunoblotted with anti-GST-p85 antiserum as in *A*. Purified GST-p85 was loaded in the far right-hand lane (−). The full length GST-p85–immunoreactive band is indicated.

of PI3-kinase. Subsequent studies were performed to determine whether the levels of PI3-kinase activities precipitated by the GST-Fyn fusion proteins reflected the amounts of p85 in these precipitates. To this end, lysates from exponentially growing CTLL-2 cells were incubated with the indicated GST-Fyn fusion proteins coupled covalently to Sepharose. Two parallel binding reactions were performed. After binding and washing, one set of precipitates was eluted with SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and immunoblotted with an anti-p85 serum (Fig. 6 *A*). PI3-kinase enzymatic assays were performed on the parallel set of precipitates (Fig. 6 *B*). The results indicated that the binding of immunoreactive p85 was well correlated with the total PI3-kinase activity precipitated by the isolated Fyn domains.

The interaction of cell-derived PI3-kinase with the SH3 domain of Fyn could occur through the regulatory p85 subunit, the catalytic p110 subunit, or via another adaptor protein present in the cell lysates. Previous work demonstrated that SH3 domains bind to a proline-rich consensus sequence (27, 28), and examination of the p85 primary amino acid sequence revealed two potential SH3 domain binding sites. Therefore, we reasoned that p85 might interact directly with the Fyn SH3 domain. To assess this possibility, the indicated GST-Fyn fusion proteins coupled covalently to Sepharose were incubated with soluble GST-p85$^{AA158-724}$. The protein precipitates were fractionated by SDS-PAGE and immunoblotted with an anti-p85 antiserum (Fig. 6 *C*). The isolated Fyn SH3 domain also displayed the highest level of p85-binding activity, although the isolated Fyn SH2 domain also displayed detectable binding activity. As predicted, the fusion protein containing both the SH2 and SH3 domains of Fyn displayed substantial binding to p85. The production of GST-p85 in *E. coli* yielded both full-length protein and a series of partial degradation products. Because these truncated fusion proteins bind to GSH-agarose, they most likely represent COOH-terminal deletions of p85, which would retain the putative SH3-binding sequences. Consequently, truncated GST-p85 proteins also complexed with the GST-Fyn fusion proteins. Thus, the interaction between purified, recombinant p85 and the Fyn SH3 domain recapitulates that observed with PI3-kinase derived from cell lysates (compare Fig. 6, *A* and *C*).

Discussion

A growing number of hematopoietic cell surface receptors appear to function through PTK-dependent signal transduction cascades (reviewed in 29). These include the coreceptors, CD4 and CD8; the multichain immune recognition receptors, such as the T cell antigen receptor, the B cell antigen receptor, and the Fcγ receptor; and, finally, members of the cytokine receptor subfamily, which includes the IL-2R. Ligation of these receptors characteristically regulates the catalytic activities of one or more nonreceptor PTKs expressed in the host cell. For example, CD4 and CD8 are physically associated with Lck (30), whereas the T cell antigen receptor complexes with Fyn (31) and ZAP70 (32). In addition, the IL-2R interacts noncovalently with the Src-family kinases, Fyn and Lck (8–10). However, the molecular mechanisms coupling these receptor-activated kinases to downstream signal-transducing molecules remain obscure.

Both Fyn and Lck undergo catalytic activation in response to IL-2R stimulation. Moreover, elegant studies have demonstrated that both the acidic and serine-rich domains of the IL-2R p70 subunit are required for the binding and IL-2–dependent activation of Lck (9). However, Lck is clearly not obligatory for IL-2–induced mitogenesis, as evidenced by the description of a Lck-deficient CTLL-2 cell line that remains fully dependent upon IL-2 for growth (11). Thus, these results indicate that, although Lck may be an important intracellular mediator of the IL-2R signal transduction cascade, other Src-family kinases expressed in T cells may serve nearly redun-

dant functions. In the case of the Lck-deficient CTLL-2 cell line, the only other highly expressed Src-family kinase is Fyn (11). Because Fyn has been demonstrated to complex with PI3-kinase in T cells (13), we therefore have focused on the potential regulatory role of the Fyn–PI3-kinase interaction in IL-2–responsive T cells.

Studies of a variety of growth factor receptor tyrosine kinases expressed by different cell types have identified two alternative, although not mutually exclusive, mechanisms for the receptor-dependent activation of PI3-kinase. According to one model, receptor-mediated p85 phosphorylation modulates PI3-kinase subcellular localization or catalytic activity. The second model proposes that association with the autophosphorylated receptor itself or other tyrosine-phosphorylated substrates activates PI3-kinase activity in the absence of detectable p85 phosphorylation (33). In some experimental systems, phosphorylation of p85 is observed only when the receptors are expressed well above physiological levels (33). Previous studies using a non-T cell line demonstrated that IL-2 mediated the tyrosine phosphorylation of p85 (34). In the present report, we have used a cytotoxic T cell line, CTLL-2, that expresses physiological numbers of functional IL-2Rs, and is wholly dependent upon IL-2 for mitogenesis. In these cells, IL-2 elicited an increase in the tyrosine phosphorylation of the p85 subunit of PI3-kinase. To define further the mechanisms of IL-2–induced p85 phosphorylation, we analyzed the ability of a recombinant GST-p85 fusion protein to complex with and function as a substrate for a CTLL-2 cell–derived, IL-2–inducible tyrosine kinase. The in vitro assay indirectly implicated Fyn as a bona fide p85 kinase in T cells.

To further investigate the role of Fyn in the regulation of PI3-kinase activity in CTLL-2 cells, wild-type Fyn was overexpressed in a Lck-deficient CTLL-2 cell line. Fyn overexpression clearly increased the levels of PI3-kinase activity immunoprecipitated from IL-2–deprived cells with an anti-Ptyr mAb, with less significant effects on the incremental increase in antibody-bound PI3-kinase activity induced by IL-2. The latter results suggest that the level of Fyn PTK activity in untransfected CTLL.lck$^-$ cells may not limit the response of these cells to IL-2. Alternatively, an intriguing possibility, which is currently under investigation in our laboratory, is that Fyn overexpression decreases the rate at which exponentially growing CTLL-2 cells enter quiescence (G_0- or G_1-phase). A Fyn-dependent alteration in the kinetics of cell cycle withdrawal during IL-2 deprivation might lead to a persistent increase in the level of PI3-kinase activity in Fyn-overexpressing, IL-2–dependent T cells. Nonetheless, the enhancing effect of Fyn overexpression on PI3-kinase activity, coupled with the demonstration of a direct interaction of p85 with Fyn, strongly implicates Fyn as an IL-2R–linked PTK that phosphorylates the downstream signal-transducing enzyme, PI3-kinase, in response to IL-2R occupancy. These conclusions do not imply that Fyn is the only PTK capable of regulating PI3-kinase activity in response to IL-2. Indeed, a recent report by Taichman et al. (26) indicated that PI3-kinase also associates with Lck in CTLL-2 cells; although previous studies from our laboratory failed to document the recovery of PI3-kinase in anti-Lck immunoprecipitates (13). Also, we have been unable to recover specifically bound lysate-derived Lck from GST-p85 fusion protein precipitates. These results indicate that, in this experimental system, Fyn is the predominant Src-family kinase to which the IL-2R and PI3-kinase are coupled. These discrepant findings might be explained by interlaboratory differences in the CTLL-2 cell lines or the polyclonal anti-Lck antibodies used to prepare the immunoprecipitates. However, earlier results clearly indicate that the IL-2R is capable of coupling to both Lck and Fyn, and it is plausible that both PTKs subserve redundant functions in the activation of PI3-kinase in response to IL-2.

While these results were prepared for publication, reports describing the interaction of potential downstream effector substrates with several different regions of the Src-family kinases were published. The results of Pleiman et al. (35) demonstrated that Ras-GTPase activating protein, phospholipase C-γ2, and MAP kinase associate with the unique NH_2-termini of Lyn, Fyn, and Blk. In addition, the SH3 domains of both Fyn and Lyn were found to associate with cell-derived PI3-kinase activity (35). Similar findings have also been reported for the SH3 domain of v-Src in a fibroblast model system (36). Our findings confirm these data, and moreover, strongly suggest that an SH3 domain–mediated interaction of Fyn with PI3-kinase functions in the regulation of PI3-kinase activity in IL-2–responsive T cells.

Taken in aggregate, these data allow the formulation of a working model for IL-2R–mediated activation of PI3-kinase. The SH3 and, possibly, the SH2 domains of Fyn bind constitutively and directly to the p85 subunit of PI3-kinase. IL-2R occupancy provokes the activation of Fyn, and, in turn, the phosphorylation of the Fyn-bound p85 subunit of PI3-kinase. This model predicts that the SH3 domains of the Src-family kinases may play central roles in the approximation of specific substrates to the catalytic domains of these PTKs. In support of this model, the SH3 domain of v-Src has been reported to bind a 110-kD protein in v-Src–transformed cells. Deletion of the SH3 domain prevents both the binding and tyrosine phosphorylation of this protein (37).

The SH3 domains of the Src-family kinases may also participate in intramolecular regulation of the catalytic activities in these enzymes. The prototypical member of this family, Src, is proposed to fold into an enzymatically repressed conformation. This repression requires both a phosphotyrosine (Y527)-SH2 domain interaction as well as an intact SH3 domain (38). Folding into the repressed conformation may sterically hinder the binding of p85 to the PTK, thereby rendering the kinase unable to interact with and phosphorylate this potential substrate. Alternatively, the interaction of other proteins with the SH3 domain of Src-family kinases may abrogate this repressive mechanism, thereby promoting the transition to an active conformation. One function of PI3-kinase interaction with Fyn may be to supply a population of partially activated Fyn molecules that would have a heightened sensitivity to IL-2R–dependent activation signals.

SH3 domains are rapidly emerging as important motifs

mediating protein-protein recognition and the assembly of molecular signaling complexes. A prototypical example of an SH3 domain–dependent regulatory cascade is provided by the recently reported association between the adaptor protein, GRB2, and the guanine nucleotide exchange factor, SOS (27). This association defines a molecular link between the growth factor tyrosine kinase receptors and a key component of the mitogenic signaling pathway, Ras. The data presented here expand upon the potential roles for the SH3 domains of Src-family kinases. In this context, the ligand-binding specificities of the SH3 domains may be a primary determinant of the spectrum of downstream effector molecules that undergo receptor-mediated tyrosine phosphorylation.

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