Activation of Phosphatidylinositol-3 Kinase by Ligation of the Interleukin-7 Receptor Is Dependent on Protein Tyrosine Kinase Activity

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Ligation of the interleukin-7 receptor (IL-7R) results in a rapid phosphorylation of tyrosine residues on multiple substrates. In addition, we have recently shown that the IL-7R mediates activation of phosphatidylinositol-3 (PI-3) kinase. Because PI-3 kinase activity can be immunoprecipitated with anti-phosphotyrosine antibodies in most receptor systems studied, it has been examined that either PI-3 kinase or an associated protein become tyrosine-phosphorylated after ligand binding. We studied here the possibility that PI-3 kinase, which is directly linked to mitogenic responses in growth factor receptors, is tyrosine-phosphorylated after stimulation of the IL-7R. Using anti-p85α or anti-p85β antibodies raised against the p85 subunit of PI-3 kinase for immunoprecipitation and subsequent blotting with antiphosphotyrosine clearly shows that IL-7-stimulated human precursor cells contain both p85α and p85β proteins phosphorylated on tyrosine residues. Specific protein tyrosine kinase inhibitors such as tyrphostin AG-490 block total cell lysate phosphorylation and tyrosine phosphorylation on p85. Similar concentrations of this inhibitor also block in vitro and in vivo PI-3 kinase activity suggesting that this enzyme activation is dependent on the phosphorylation event of p85. In addition, AG-490 blocks IL-7-mediated proliferation in a dose-dependent manner, suggesting a link between the early events of PI-3 kinase phosphorylation and activation with IL-7R-induced cell growth.

INTERLEUKIN-7 (IL-7) is a 25-kD glycoprotein involved in the regulation of lymphocyte growth. Originally defined by its ability to stimulate the proliferation and differentiation of pre-B cells, IL-7 was subsequently shown to affect the growth of cells of T-cell lineage.1-3 Resting fetal and adult thymocytes proliferate in response to IL-7 independent of IL-2, IL-4, and IL-6.4,5 Mature resting T cells are also induced to synthesize DNA by IL-7 in the presence of suboptimal mitogen concentrations6 or in combination with phorbol-12-myristate 13-acetate.7 Recently, cDNA encoding the IL-7 receptor (IL-7R) have been cloned, and analysis of the sequences showed homology in the extracellular domain to other members of the hematopoietin receptor family, including several cytokine receptors as well as the receptors for growth hormone and prolactin.8,9 However, the cytoplasmic domain of the IL-7R showed some sequence homology to human growth hormone receptor and IL-2R β chain.

Although a wealth of knowledge has been accumulated concerning the structure distribution and biologic functions of cytokine receptors, the mechanism by which these receptors transduce signals remains unresolved. Recently, we have shown that IL-7 binding induces the activation of an IL-7R-associated tyrosine kinase.10 Protein tyrosine kinase (PTK) activation results in phosphorylation of various substrates within 1 to 2 minutes of ligand binding, and it appears critical to the biologic action of IL-7.11 Yet another biochemical event occurring after ligation of the IL-7R is the activation of phosphatidylinositol-3 (PI-3) kinase.12 PI-3 kinase phosphorylates PI, PI 4-phosphate (PI4P), and PI 4,5 bisphosphate (PI4,5P2) at the D-3 position of the inositol ring13 to produce the novel products, PI 3 phosphate (PI3P), PI 3,4 bisphosphate (PI3,4P2), and PI 3,4,5 trisphosphate (PI3,4,5P3), respectively. The product PI3P is not involved in the classical pathway of generating second messengers such as inositol trisphosphate but was reported to activate protein kinase C.14 PI-3 kinase associates with PTK receptors such as platelet-derived growth factor (PDGF)15 and insulin16 and with cytoplasmic tyrosine kinases such as the polyoma middle-T-activated pp60v-SRC. In these receptor systems, PI-3 kinase activity was identified in antiphosphotyrosine immune complexes, suggesting that either the enzyme or an associated protein are phosphorylated on tyrosine residues on stimulation of the receptor.

We have shown here that ligation of the IL-7R in lymphocyte progenitor cells is associated with tyrosine phosphorylation on p85, a subunit of PI-3 kinase. Such increase in PTK activity induced by the IL-7R seems to be essential for activation of PI-3 kinase, as shown by experiments performed in the presence of the specific PTK inhibitors, tyrphostins. Tyrphostin AG-490, which blocks tyrosine phosphorylation mediated by IL-7, also inhibited the activation of PI-3 kinase and IL-7-induced cell proliferation.

MATERIALS AND METHODS

Cell preparation and cell lines. Thymuses were obtained from children who had open heart surgery. Mononuclear cells were isolated by Ficoll-Hypaque gradient. Adherent cells were removed by adherence to plastic dishes for 60 minutes at 37°C. The resulting thymocyte population expressed less than 1% B1 cells and greater than 98% CD3 cells. v-Src-transformed Rat 2 fibroblast cells were provided by Dr T. Pawson (Mount Sinai Hospital, Toronto, Canada). Pre-pre-B-cell line G2 was derived from a patient with acute lymphocytic leukemia and was phenotypically characterized.17 This line expresses CD10, CD19, and CD72, whereas surface Ig and cytoplasmic μ chains were not detected. All determinations of surface markers were made by immunofluorescence staining on a flow cytomter (Epics V; Coulter Electronics, Hialeah, FL).

Reagents and antibodies. PI, PI 4,5-phosphate (PI4P), and PI4P were purchased from Sigma Chemical Co (St Louis, MO). The radiolabeled reagents (γ32P-ATP, >5,000 Ci/mmol; 3H-PI4P, 1.0 Ci/mmol; 3H-PI4,5P2, and 125I-Protein A, >30 mCi/mg) were from Amersham (Arlington Heights, IL).

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Recombinant human IL-7 (rhIL-7) was purchased from GIBCO, BRL (Gaithersburg, MD), and the Silica Gel 60 TLC plates were from Merck (Darmstadt, Germany). Anti-p85α and anti-p85β antibody raised against the p85 subunit of PI-3 kinase were a kind gift of Drs. I. Got and M. Waterfield (Ludwig Institute for Cancer Research, London, UK). Monoclonal antiphosphotyrosine IgG 2bk and Genistein were purchased from Upstate Biotechnology Inc (New York, NY). Rabbit antimouse (RAM) IgG was purchased from Western Blotting Enterprises (Toronto, Ontario, Canada).

Tyrosinase blocker AG-490 and AG-126 were kindly provided by D.A. Levitzki (Department of Biological Chemistry, Hebrew University of Jerusalem, Jerusalem, Israel). Tyrosinases were derived from benzylidenedenamalononitrile nucleus. The guidelines for selection of compounds included compounds soluble in water as well as in mildly hydrophobic solvents. Such solubility properties allow the compounds to traverse the cell membrane of intact cells. All other chemicals were from BDH (Toronto, Ontario, Canada) or Sigma.

Western blot. Cells (4 x 10⁶) were incubated in the presence or absence of various ligands as indicated. Control cells were incubated with the appropriate excipient buffer control. Western blotting was performed essentially as described. Briefly, cells were pelleted, and the reaction was stopped by lysing cells in 2x sodium dodecyl sulfate (SDS)-gel sample buffer, followed by immediate boiling for 5 minutes. Lysates were centrifuged at 12,000 g for 5 minutes, and supernatants were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were electrotransferred onto Nitrocellulose membranes and blocked overnight with 5% bovine serum albumin (BSA; essentially fatty acid and globulin free; Calbiochem Inc, San Diego, CA) in 10 mmol/L Tris, 150 mmol/L NaCl, pH 8.0 (TBS). The blot was incubated with 3 μg of affinity-purified phosphotyrosine-specific mouse antibodies in 4 mL of TBS containing 1% BSA followed by 1-hour incubation with RAM IgG. This was followed by 3 10-minute washes with TBS containing 0.05% Tween 20 (TBST). The filters were then incubated with 1 μCi of [32P]-labeled protein A in 5 mL of TBST containing 1% BSA for 1 hour and were washed as before. The filters were then autoradiographed.

Stimulation and lysis of cells. Cells, 2 x 10⁶ cells/mL in RPMI 1640 growth medium, were stimulated with 30 ng/mL of rhIL-7 for 15 minutes at 37°C. Cells were pelleted by centrifugation (14,000 g, 10 seconds) and lysed in 1 mL of lysis buffer (20 mmol/L Tris, pH 7.5; 150 mmol/L NaCl; 5 mmol/L EDTA; 1% NP-40; 1 mmol/L sodium orthovanadate; and 1 mmol/L phenylmethylsulfonyl fluoride [PMSF]) for 15 minutes at 4°C. The ability of tyrosinases to block PI-3 kinase activity was tested by incubating thymocytes in the presence or absence of 100 μmol/L tyrosinase or 30 μg/mL of genestein for 16 hours before stimulation with rhIL-7.

Immunoprecipitation and enhanced chemiluminescence (ECL) blotting. For 10 assays, 25 mg of Protein-A sepharose CL-4B was washed 3 times with lysis buffer without PMSF and was resuspended in 1 mL of the same buffer. A total of 100 μg of RAM IgG was added to the bead suspension and incubated with mixing for 2 hours at 4°C. The antibody-sepharose conjugate was then washed 3 times with lysis buffer, was resuspended in 1 mL buffer containing 30 μg of antiphosphotyrosine IgG 2bk, and was incubated with mixing for 2 hours at 4°C. The beads were then washed 3 times with lysis buffer and resuspended in the same buffer to give a 50% suspension. To the cell lysates was added 15 μL of the sepharose-antiphosphotyrosine antibody conjugate, and the mixture was incubated for 16 hours at 4°C.

The immunoprecipitates were washed 5 times with lysis buffer, and an equal volume of 2x SDS-PAGE sample buffer was added. This was followed by boiling for 5 minutes and separation by SDS-PAGE.

The separated proteins were electrotransferred onto Nitrocellulose membranes and blocked as described above. After incubation with primary antibody, the blot was washed with TBST at 4°C for 10 minutes before incubation with 1:1,000 dilution of horseradish peroxidase-conjugated species-specific Ig (Amersham) for 1 hour at 4°C. After washing the blot was developed with the ECL system (Amersham) according to the manufacturer's instructions.

Pl-kinase activity. PI-kinase activity was measured as described by Fukui and Hanafusa. The immunopellets were washed 3 times with lysis buffer, once with phosphate-buffered saline, once with 0.5 mol/L lithium chloride and 0.1 mol/L Tris (pH 7.5), once with double distilled water (dH₂O), and twice with PI-kinase buffer (20 mmol/L Tris, pH 7.5; 100 mmol/L NaCl; 0.5 mmol/L EGTA) with or without 1% NP-40. The immunopellets were then resuspended in 50 μL of PI-kinase buffer.

PI (20 mg) sonicated in 1 mL of diethyl sulfoxide was added to the immunopellets to a final concentration of 0.2 mg/mL; it was then vortexed and incubated at 25°C for 10 minutes. The phosphory-

Fig 1. Effect of tyrphostin and genistein on IL-7-induced tyrosine phosphorylation. Thymocytes (4 x 10⁷ cells) were incubated for 16 hours with or without tyrphostin AG-490, AG-126, or genistein and were subsequently stimulated with rhIL-7 or antibody against major histocompatibility complex class F for 15 minutes. Whole cell lysates were prepared as described in Materials and Methods and analyzed by SDS-PAGE. After electrophoresis and blotting, the phosphotyrosine-containing proteins were detected using phosphotyrosine antibodies and [32P]-labeled protein A. The resulting autoradiogram is shown. The data represent one of four different experiments with similar results.
lation reaction and extraction of lipids was according to the method of Whitman et al.14 Phosphorylation was initiated by the addition of 25 mmol/L MgCl₂ and 5 μCi (³²P)ATP. After 10 minutes at 25°C, the reaction was stopped by the addition of 150 μL of CHCl₃:MeOH:H₂O (43:38:5:7 [vol/vol/vol]), and the lipids were extracted with 150 μL of CHCl₃. The organic phase was washed with MeOH:1 mol/L HCl (1:1 [vol/vol]), and the lipid sample was dried at 60°C for 15 minutes.

The lipids were resuspended in 10 μL of CHCl₃ , spotted on thin layer chromatography (TLC) silica gel 60 plates, and developed in CHCl₃:MeOH:4 mol/L NH₄OH:H₂O (43:38:5:7 [vol/vol/vol/vol]), and the phosphate incorporation was visualized by autoradiography of the dried plates. ³²P incorporation into PIP was quantitated by scraping the radiolabeled spots from the TLC plates and by counting in a Beckman LS 3801 β-counter (Beckman Instruments, Irvine, CA).

In vivo labeling of cells. In vivo labeling was performed according to Remillard, et al.15 Thymocytes in phosphate-free RPMI were labeled with 0.1 mCi/10⁶ cells/mL of ³²PO₄ for 3 hours before stimulation with 30 ng/mL of rhlL-7 for 15 minutes at 37°C. Cells were washed with RPMI, and the lipids were extracted into 1 N HCl:chloroform:methanol (1:2:1 [vol/vol/vol]) and deacylated as described below. The products were separated by high performance liquid chromatography (HPLC) and identified by comparison with deacylated ¹H P14P and ¹HPI4.5P standards.

HPLC analysis of the PI-kinase reaction products. PIP reaction products were scraped from TLC plates and deacylated to their glycerol-inositol phosphate forms by incubation with 400 μL of 10.88% methylamine solution (10.8% methylamine, 32.1% H₂O, 45.7% MeOH, 11.4% nBuOH) for 50 minutes at 53°C.16 The deacylated lipid mixture was dried in vacuo, redissolved in 1 mL of H₂O, and extracted twice with butanol:petroleum ether:ethyl formate (20:4:1 [vol/vol/vol]).15 The aqueous phase was dried in vacuo, redissolved in 250 μL of H₂O, and mixed with a ¹H-P14P standard (similarly deacylated to its glycerol-phosphate form) and analyzed on HPLC using a Partisil SAX-5 column (Whatman Inc., Maidstone, UK).

Samples were loaded in H₂O for 10 minutes and eluted with a linear gradient11 of 0.01 mol/L to 0.25 mol/L (NH₄)H₂PO₄, pH 3.8, over 60 minutes followed by a linear gradient of 0.25 mol/L to 1 mol/L (NH₄)H₂PO₄ over 50 minutes [pump A, H₂O; pump B, 1 mol/L (NH₄)H₂PO₄] with a flow rate of 1 mL/min, and 0.5 mL fractions were collected and counted for ¹H and ³²P.

RESULTS

Ligation of IL-7R results in a rapid increase of PTK activity. We used Western blotting (Fig 1) with antiphosphotyrosine antibodies to detect rhlL-7-induced tyrosine phosphorylation in human thymocytes. In full agreement with previous observations, we have consistently observed that rhlL-7 induced a time- and concentration-dependent increase in tyrosine phosphorylation of multiple proteins, including major bands with an apparent molecular weight of 110 kD and 85 kD,14 whereas ligation of major histocompatibility complex (MHC) class I molecules had no effect. Tyrosine phosphorylation was detected within 1 minute of addition of rhlL-7, with maximal increases observed 10 to 15 minutes later.14 Phosphorylation detected on these Western blots were strictly on tyrosine residues because antiphosphotyrosine antibodies used have been shown to react specifically with phosphorytrosine and not with phosphoserine or phosphothreonine.22,23 Further,more, PTK inhibitors such as genestein completely abrogated tyrosine phosphorylation induced by IL-7. In addition, we have used tyrphostins, cis-benzemalononitrile derivatives that were previously found to be specific PTK inhibitors. We have identified one tyrphostin, AG-490, that specifically blocks IL-7R-mediated responses in a dose-dependent fashion but has no effect on epidermal growth factor (EGF)-, PDGF-, or antigen receptor-mediated tyrosine phosphorylation (data not shown). Tyrphostin AG-126, an efficient blocker of B-cell--antigen receptor-mediated responses,22 had no inhibitory effect on IL-7-induced tyrosine kinase activity (Fig 1), showing that some of these analogues have a specific affinity to various PTKs. Tyrphostin AG-490 did not exact its effect by altering IL-7 binding to its receptor or by affecting IL-7 expression, as shown by experiments where-by IL-7 fluorokine binding to its receptor in the presence or absence of AG-490 was analyzed by flow cytometry. No difference in binding was detected between AG-490 untreated cells (18%) and treated thymocytes (19%; data not shown).

Fig 2. Effect of rhlL-7 on tyrosine phosphorylation of p85. Thymocytes (A) or pre-B cells (B) (2 × 10⁵) were incubated with or without rhlL-7 (30 ng/mL). The incubations were terminated after 15 minutes by pelleting the cells and solubilizing them in lysis buffer. The lysates were immunoprecipitated with antiphosphotyrosine antibodies, anti-p85α, or anti-p85β antibody as indicated. The immunopellets were solubilized in SDS-PAGE sample buffer and electrophoretically separated on an 8% SDS-polyacylamide gel. After Western blotting, the phosphotyrosine-containing proteins were detected using phosphotyrosine antibodies, whereas the p85 subunit of PI-3 kinase was detected using the antibody to p85 and ECL.
We could therefore conclude that rhIL-7 consistently induced tyrosine phosphorylation in lymphoid precursor cells.

Protein tyrosine phosphorylation of PI-3 kinase in lymphoid precursor cells. Ligation of the IL-7R of B- and T-cell precursor cells results in a rapid increase of tyrosine phosphorylation on multiple substrates. To date, we have been able to identify some of these substrates, we have previously shown that IL-7 did not increase phosphorylation and activation of phospholipase C-γ. Consequently, IL-7 failed to increase cytosolic free Ca\(^{2+}\) or inositol phosphate production. Therefore, we could conclude that the IL-7R did not use the classical PI-4 kinase for signal transduction. Instead, the predominant phosphorylation of an 85-kD protein by stimulated IL-7R raised the possibility that yet another PI kinase, PI-3 kinase, might be linked to this receptor system. This recently identified PI-3 kinase was shown to be closely linked to cell proliferation and transformation through various receptor systems (for review see 28), and consists of two subunits of 81 to 85 kD and 110 kD.\(^{30,32}\) The fact that PI-3 kinase activity can be identified in antiphosphotyrosine immune complexes obtained from many cell types raises the possibility that either p85 or an associated protein are phosphorylated on tyrosine residues.

To identify whether the IL-7R mediates the phosphorylation of PI-3 kinase we have used anti-p85α and anti-p85β antibodies\(^{32}\) to immunoprecipitate lysates of unstimulated and rhIL-7-stimulated cells.

Immunoprecipitation and subsequent immunoblotting with the same antibody anti-p85α and anti-p85β confirmed the presence of both isomers in lymphocytes (Fig 2). Immunoprecipitation with anti-p85α or anti-p85β and subsequent blotting with antiphosphotyrosine antibody showed that both isomers become phosphorylated on tyrosine residues shortly after ligation of the IL-7R on thymocytes (Fig 2A). In full agreement with these results, tyrosine-phosphorylated p85α or p85β were also detected in leukemia cells representing...
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B-lineage progenitor cells (Fig 2B). Similar experiments performed on cells pretreated for 6 hours with genestein showed complete inhibition of tyrosine phosphorylation on p85α (Fig 2B) or p85β (data not shown).

The results provide direct evidence that p85α and p85β are tyrosine-phosphorylated after ligand binding.

PI-3 kinase activity associated with the IL-7R is dependent on tyrosine kinase activity. To define the link between PTK activity and PI-3 kinase, we have used two strategies. First, PI-3 kinase activity was measured in antiphosphotyrosine immune complexes, and, second, the effect of specific PTK inhibitors on IL-7R-associated PI-3 kinase activity was studied.

To detect PI-3 kinase in antiphosphotyrosine complexes, thymocytes pretreated with either medium or PTK inhibitors for 16 hours were incubated for 15 minutes with either media or rhIL-7 at a concentration that was found to be optimal for tyrosine phosphorylation and proliferation. Cells were subsequently lysed and immunoprecipitated with antiphosphotyrosine antibody. Immune complexes were then suspended in a reaction buffer containing PI and [γ-32P]ATP. IL-7R-associated PI-kinase activity was indirectly determined by detecting the enzyme reaction product, [32P]PIP. [32P]PIP was separated from PI and [γ-32P]ATP by TLC and quantified by autoradiography. Figure 3 shows a marked increase in [32P]PIP in the pre-B-cell sample (Fig 3A) or the thymocyte (Fig 3B) sample treated with rhIL-7 over control untreated sample. The magnitude of response was similar to CTLL-2 cells treated with IL-2 (results not shown) or to the v-src-transformed Rat 2 fibroblast cell line.

To examine the specificity of the PI-kinase activity, we first tested its susceptibility to inhibition by the nonionic detergent NP-40.33 Such detergents were previously shown to inhibit PI-3 kinase activity and to enhance PI-4 kinase activity.25 Figure 3B shows the effect of NP-40 on PI-3 kinase activity in IL-7-stimulated thymocytes. At 1%, NP-40 the kinase reaction was completely abolished. Similar concentrations of NP-40 also inhibited PI-3 kinase activity in IL-2-treated CTLL-2 cells and in other receptor systems.33 Direct detection of PI3P in the kinase reaction products was performed by HPLC analysis. The lipid was retrieved from the TLC plate and was deacylated to yield [32P]-labeled glycerol-phosphate products, gPI3P and gPI4P, which were then separated on an anion exchange column.16 [32P]gPI3P eluted at 30 minutes, whereas [3H]gPI4P eluted at 33 minutes. The major deacylated product in the rhIL-7-treated sample comigrated with gPI3P (not shown).

To determine whether IL-7R-associated PI-3 kinase activity is dependent on PTK activity, we have studied the effects of tyrphostins and genistein, inhibitors of tyrosine kinases, on IL-7-stimulated cells. As shown in Fig 3B, pre-treatment of thymocytes with optimal concentrations of AG-490 (100 μmol/L) or genistein completely blocked the ligand-induced increase in PI-3 kinase activity. In complete agreement with previous reports,22,23,37 tyrphostins used at these concentrations had no deleterious effect on cell viability. Other tyrphostins, such as AG-126 that can block antigen receptor-mediated PTK activity25 or AG-186 that blocks EGF receptor activity, had no inhibitory effect on IL-7R-associated PI-3 kinase activity (not shown). Further, AG-490 had no inhibitory effect on B-cell antigen receptor-mediated PI-3 kinase activity (Fig 3C), indicating that this compound does not directly block PI-3 kinase. The results indicate that AG-490 specifically blocks an IL-7-associated PTK rather than directly affecting PI-3 kinase activity as shown in Fig 4.

Inhibition of PI-3 kinase activity by AG-490 was concentration-dependent and correlates well with AG-490 dose response on p85 phosphorylation (Fig 4). This dose response further supports the idea that IL-7R-mediated PI-3 kinase activity is dependent on tyrosine phosphorylation of p85.

Such a link was supported by the kinetics of response to rhIL-7 for tyrosine phosphorylation and PI-3 kinase activity. We have previously shown that optimal concentrations of IL-7 (1 nmol/L) induced tyrosine phosphorylation that was detected within 1 minute, with maximum response at 10 to 15 minutes.14 Similarly, PI-3 kinase activity was detected within 1 to 2 minutes of rhIL-7 stimulation, with maximum activity after 10 minutes.15 Such maximal PI-3 kinase activity was achieved by incubating thymocytes with 0.5 to 1 nmol/L of rhIL-7 as shown previously by the dose-dependent curve.15 The results thus suggest that activation of PI-3 kinase is dependent on receptor-mediated tyrosine phosphorylation.

Effect of PTK inhibitors on IL-7R-mediated PI-3 kinase activity. To directly determine whether PI-3 kinase activation by the IL-7R is dependent on PTK activity, we have determined the accumulation of PI-3 kinase products in intact cells (in vivo) stimulated with IL-7 in the presence or
absence of tyrphostins. If PI-3 kinase activity is dependent on PTK activity as suggested by the in vitro studies, then AG-490 should block PI-3 kinase activation in vivo. To study this possibility, thymocytes were pretreated with medium or AG-490 for 16 hours and subsequently metabolically labeled with $^{32}$P$^{32}$O. Polyphosphoinositides were then analyzed by HPLC. Figure 5 shows a significant increase in D-3 phosphorylated polyphosphoinositides in IL-7-stimulated cells compared with that for unstimulated controls. In addition, the formation of the PI-3 kinase products, in thymocytes pretreated with the AG-490 tyrophostin, metabolically labeled with $^{32}$P$^{32}$O, and stimulated with rhIL-7, was analyzed on HPLC. The results in Fig 5 show that all three phosphorylated polyphosphoinositides that are products of PI-3 kinase activation are significantly inhibited by the tyroprotin blocker AG-490, thus indicating that tyrosine phosphorylation is required for the activation of PI-3 kinase.

**Effect of AG-490 on IL-7-mediated thymocyte growth.** IL-7 is a potent cytokine promoting the growth and maturation of progenitor cells of both B- and T-cell lineage. To test whether the early events such as tyrosine phosphorylation and PI-3 kinase activity are relevant signals for thymocyte growth, we have measured $[^{3}H]$thymidine incorporation in IL-7-stimulated thymocytes pretreated with AG-490 or AG-126.

AG-490 but not AG-126 inhibited IL-7-mediated proliferation in a dose-dependent manner (Fig 6). The kinetic of AG-490 inhibitions of thymocyte growth, correlated with inhibition of tyrosine phosphorylation and PI-3 kinase activity, suggesting that these early events are important for the transduction of growth signals through the IL-7R.

**DISCUSSION**

In the present study, we showed that phosphotyrosine immunoprecipitates obtained from IL-7-stimulated human lymphocyte precursor cells contained PI-3 kinase activity. This recently discovered PI kinase phosphorylates polyphosphoinositides at the D-3 position of the inositol ring. The function of such inositol lipids that are phosphorylated at the D-3 position remains unknown, but they do not appear...
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In contrast, similar experiments performed with anti-p110α/β antibody showed no increase in phosphorylation (data not shown). These studies directly show, for the first time, that p85 is contained both p85α and p85β under similar conditions. The PI-3 kinase is a strongly associated heterodimer of the 110-kD and the 85-kD proteins. Immunoprecipitation with anti-p85α or anti-p85β and subsequent immunoblotting with antiphosphotyrosine antibody showed an increase in tyrosine phosphorylation of both isoforms after IL-7R stimulation. Furthermore, antiphosphotyrosine immune complexes contained both p85α and p85β under similar conditions. In contrast, similar experiments performed with anti-p110 showed no increase in phosphorylation (data not shown). These studies directly show, for the first time, that p85 is tyrosine-phosphorylated. Tyrosine phosphorylation on p85 could only be detected by using the very sensitive ECL method, suggesting that this phosphorylation event involves only a small number of tyrosine residues.

To determine whether tyrosine phosphorylation is required for activation of PI-3 kinase through the IL-7R, we have used PTK blockers, tyrphostin AG-490 and genistein. Tyrphostin AG-490 was found to be most effective in blocking IL-7-mediated tyrosine phosphorylation, as was genistein. In the presence of similar doses of the inhibitor, tyrosine phosphorylation of PI-3 kinase was efficiently inhibited. This inhibitory effect of AG-490 seems to have been specific rather than nonspecific toxicity because cell viability remained intact. If tyrosine phosphorylation is required for PI-3 kinase activation, then similar doses of AG-490 should block IL-7R-mediated PI-3 kinase activity.

To study PI-3 kinase activation by the IL-7R, we first determined whether antiphosphotyrosine immunoprecipitates obtained from human thymocytes possessed PI-3 kinase activity. PI-3 kinase activity was determined by using an in vitro assay that measures the incorporation of $^{32}$P into PI to yield [$^{32}$P]P3P (Fig 3), the identity of which was confirmed by HPLC analysis. Increased activity was detected after stimulation with IL-7, with kinetics and dose response similar to IL-7-induced tyrosine phosphorylation. IL-7-induced PI-3 kinase activity was distinguished from PI-4 kinase by the addition of a nonionic detergent such as NP-40. This detergent was previously shown to preferentially inhibit PI-4 kinase but not PI-3 kinase activity. The identification of IL-7-dependent PI-3 kinase activity was also directly confirmed by detection of deacylated D-3-phosphorylated polyphosphoinositides from intact thymocytes on HPLC (Fig 4). Pretreatment of cells with AG-490 completely abolished PI-3 kinase activity stimulated by IL-7 (Fig 3 and 4). Furthermore, inhibition of p85 phosphorylation and PI-3 kinase activity were dependent on AG-490 concentration. The kinetics of inhibition of these events were very similar suggesting that IL-7R-mediated PI-3 kinase activity is dependent on p85 phosphorylation. In addition, similar concentrations of AG-490 also blocked IL-7-induced tyrosine phosphorylation (Fig 6), suggesting that the early event of PTK-dependent PI-3 kinase activity is required for IL-7R-mediated growth signaling.

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Activation of phosphatidylinositol-3 kinase by ligation of the interleukin-7 receptor is dependent on protein tyrosine kinase activity

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