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Research Article

Interleukin-7 (IL-7) is a glycoprotein that regulates lymphocyte precursor growth and differentiation. However, the exact mechanism whereby the IL-7 receptor (IL-7R) mediates these cell growth signals remains unknown. One of the earliest metabolic events linked to mitogenic responses in other growth factor receptor systems is the activation of phosphatidylinositol-3 kinase (PI-3 kinase). We demonstrate here that ligation of the IL-7R results in dose- and time-dependent increases in PI-3 kinase activity. These results suggest that PI-3 kinase is involved in signal transduction via the IL-7R in human thymocytes.

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Activation of Phosphatidylinositol-3 Kinase by Ligation of the Interleukin-7 Receptor on Human Thymocytes

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Abstract

Interleukin-7 (IL-7) is a glycoprotein that regulates lymphocyte precursor growth and differentiation. However, the exact mechanism whereby the IL-7 receptor (IL-7R) mediates these cell growth signals remains unknown. One of the earliest metabolic events linked to mitogenic responses in other growth factor receptor systems is the activation of phosphatidylinositol-3 kinase (PI-3 kinase). We demonstrate here that ligation of the IL-7R results in dose- and time-dependent increases in PI-3 kinase activity. These results suggest that PI-3 kinase is involved in signal transduction via the IL-7R in human thymocytes. (*J. Clin. Invest.* 1993;92:1559–1563.) Key words: phosphatidylinositol • phosphatidylinositol 3 phosphate • phosphatidylinositol 4 phosphate • recombinant human interleukin-7

Introduction

IL-7 is a 25-kD glycoprotein involved in the regulation of lymphocyte growth. Originally defined by its ability to stimulate the proliferation of pre-B cells, IL-7 was subsequently shown to affect the growth of cells of T cell lineage (1–8). Resting fetal and adult thymocytes proliferate in response to IL-7 independent of IL-2, IL-4, and IL-6 (9, 10). Mature resting T cells are also induced to synthesize DNA by IL-7 in the presence of suboptimal mitogen concentrations (7), or in combination with phorbol-12-myristate 13-acetate (11). Recently, cDNA encoding the IL-7R have been cloned, and analysis of the sequences revealed 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 (12, 13). However, the cytoplasmic domain of the IL-7R showed some sequence homology to human growth hormone receptor and IL-2R β chain.

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1. *Abbreviations used in this paper:* PI, phosphatidylinositol; PI₄,5P₂, phosphatidylinositol 4,5 biphosphate; PI-3 kinase, phosphatidylinositol-3 kinase; PIP, phosphatidylinositol phosphate; rhIL-7, recombinant human interleukin-7.

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While a wealth of knowledge has been accumulated concerning the structure distribution and biological 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 (14). Protein tyrosine kinase activation results in phosphorylation of various substrates within 1–2 min of ligand binding, and it appears critical to the biological action of IL-7 (14). To date the identity of these substrates remains elusive. We have previously shown that the putative IL-7R-linked protein tyrosine kinase is not using phospholipase C as a substrate. We studied here the possibility that the IL-7R might activate yet another phosphoinositide cascade regulated by phosphatidylinositol-3 kinase (PI-3 kinase)¹ that has been linked to the tyrosine phosphorylation pathway. In all receptor systems studied to date, PI-3 kinase activity was identified in anti-phosphotyrosine immune complexes, suggesting that either the enzyme or an associated protein are phosphorylated on tyrosine residues upon stimulation of the receptor.

PI-3 kinase phosphorylates phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PI₄P), and phosphatidylinositol 4,5 biphosphate (PI₄,5P₂) at the D-3 position of the inositol ring (15) to produce the novel products, phosphatidylinositol 3 phosphate (PI₃P), phosphatidylinositol 3,4 biphosphate (PI₃,4P₂), and phosphatidylinositol 3,4,5 trisphosphate (PI₃,4,5P₃), respectively. PI-3 kinase associates with the PDGF (16), insulin (17), and IL-2Rs (18) with the polyoma middle T-activated pp60^{c-src} (19) and pp68^{l-ros} (20). Mutants of middle T antigen that do not associate with PI-3 kinase failed to induce transformation (21, 22). In a similar fashion PDGF receptor mutants that did not associate with PI-3 kinase failed to mediate a mitogenic response to its corresponding ligand (23). These studies indicate that in some growth factor receptor systems, PI-3 kinase is an essential component in a signal transduction cascade leading to mitogenesis.

We have explored here the possibility that the IL-7R on thymocytes, which mediates proliferation of these cells, is linked to PI-3 kinase.

Methods

Cell preparation and cell lines. Thymuses were obtained from children having open heart surgery. Mononuclear cells were isolated by Ficoll-Hypaque gradient. Adherent cells were removed by adherence to plastic dishes for 60 min at 37°C. The resulting thymocyte population expressed < 1% B1⁺ and > 98% CD3⁺ cells. v-src-transformed rat 2 fibroblast cells were provided by Dr. T. Pawson, Mt. Sinai Hospital, Toronto.

Reagents and antibodies. PI, phosphatidylinositol 4,5-phosphate, and phosphatidylinositol 4 phosphate were purchased from Sigma

Chemical Co. (St. Louis, MO). The radiolabeled reagents, γ -[^{32}P]-ATP > 5000 Ci/mmol; [^3H]PI4P 1.0 Ci/mmol [^3H]PI4,5P₂, [^3H]inositol 1,4 bisphosphate, [^3H]inositol 1,4,5 trisphosphate were from Amersham (Buckinghamshire, UK).

Human recombinant interleukin-7 (rhIL-7) was purchased from GIBCO BRL (Gaithersburg, MD) and the silica gel 60 TLC plates were from Merck (Rahway, NJ). Monoclonal anti-phosphotyrosine IgG 2bk was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-mouse IgG was purchased from Western Blotting Enterprises (Toronto, Ontario, Canada). All other chemicals were from BDH Chemicals (Toronto, Ontario) or Sigma Chemical Co.

Stimulation and lysis of cells. Thymocytes, 2×10^7 cells/ml in RPMI 1640 growth medium, were stimulated with 30 ng/ml of rhIL-7 for 15 min at 37°C. Cells were pelleted by centrifugation (14,000 g, 10 s) and lysed in 1 ml of lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 1 mM sodium orthovanadate, and 1 mM PMSF) for 15 min at 4°C.

Immunoprecipitation. For 10 assays, 25 mg of protein A-Sepharose CL-4B were washed three times with lysis buffer without PMSF and resuspended in 1 ml of the same buffer. 100 μg of rabbit anti-mouse (RAM) IgG was added to the bead suspension and incubated with mixing, for 2 h at 4°C. The antibody-Sepharose conjugate was then washed three times with lysis buffer, resuspended in 1 ml buffer containing 30 μg of anti-phosphotyrosine IgG 2bk, and incubated with mixing for 2 h at 4°C. The beads were then washed three 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-anti-phosphotyrosine antibody conjugate and the mixture incubated for 16 h at 4°C.

PI kinase activity. PI kinase activity was measured as described by Fukui and Hanafusa (24). The immunopellets were washed three times with lysis buffer, once with PBS, once with 0.5 M lithium chloride, 0.1 M Tris, pH 7.5, once with d.H₂O and twice with PI kinase buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EGTA) with or without 1% NP-40, and resuspended in 50 μl of PI kinase buffer.

PI (20 mg) sonicated in 1 ml of DMSO was added to the immunopellets to a final concentration of 0.2 mg/ml, vortexed, and incubated at 25°C for 10 min. The phosphorylation reaction and extraction of lipids was according to the method of Whitman et al. (15). Phosphorylation was initiated by the addition of 25 mM MgCl₂ and 5 μCi [^{32}P]-ATP. After 10 min at 25°C the reaction was stopped by the addition of 150 μl of CHCl₃:MeOH:11.6 M HCl (100:200:2 vol/vol per vol) and the lipids extracted with 150 μl of CHCl₃. The organic phase was washed with MeOH:1M HCl (1:1 vol/vol) and the lipid sample dried at 60°C for 15 min.

The lipids were resuspended in 10 μl of CHCl₃ and spotted on TLC silica gel 60 plates, developed in CHCl₃:MeOH:4M NH₄OH:H₂O (43:38:5:7 vol/vol/vol/vol) and the phosphate incorporation visualized by autoradiography of the dried plates. ^{32}P incorporation into PIP was quantitated by scraping the radiolabeled spots from the TLC plates and counting, in an LS 3801 β -counter (Beckman Instrs., Inc., Fullerton, CA).

In vivo labeling of thymocytes. In vivo labeling was carried out according to the method described by Remillard et al. (25). Thymocytes in phosphate-free RPMI were labeled with 0.1 mCi/ 10^6 cells/ml of $^{32}\text{PO}_4^{3-}$ for 3 h before stimulation with 30 ng/ml of rhIL-7 for 15 min at 37°C. Cells were washed with RPMI and the lipids extracted

deacylated as described below. The products were separated by HPLC and identified by comparison with deacylated [^3H]PI4P and [^3H]PI4,5P₂ 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 methylamine solution (10.8% methylamine; 32.1% H₂O; 45.7% MeOH; 11.4% nButOH) for 50 min at 53°C (15).

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 per vol) (15). The aqueous phase was dried in vacuo, redissolved in 250 μl of H₂O, and mixed with a [^3H]PI4P standard (similarly deacylated to its glycerol-phosphate form) and analyzed on HPLC using a Partisphere Sax-5 column (Whatman Inc., Clifton, NJ).

Samples were loaded in H₂O for 10 min and eluted with a linear gradient (15) of 0.01–0.25 M (NH₄)H₂PO₄, pH 3.8, over 60 min followed by a linear gradient of 0.25–1 M (NH₄)H₂PO₄ over 50 min (pump A: H₂O; pump B: 1 M [NH₄]H₂PO₄) with a flow rate of 1 ml/min and 0.5 ml fractions were collected and counted for ^3H and ^{32}P .

Results

To study whether the IL-7R is linked to PI-3 kinase we first examined in vitro the activity of PI-3 kinase in unstimulated and rhIL-7-stimulated thymocytes. Cells were incubated for 15 min 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 anti-phosphotyrosine antibody. Immune complexes were then suspended in a reaction buffer containing PI and γ -[^{32}P]-ATP. PI-kinase association was indirectly determined by detecting the enzyme reaction product, [^{32}P]PIP. [^{32}P]PIP was separated from PI and γ [^{32}P]ATP by TLC and quantified by autoradiography. Fig. 1 shows a marked increase in [^{32}P]PIP in the sample treated with rhIL-7 over control untreated sample. The magnitude of response was similar to CTLL-2 cells treated with rhIL-2 (results not shown) or to the v-src-transformed rat 2 fibroblast cell line, shown here.

To examine the specificity of the PI kinase activity we first tested its susceptibility to inhibition by the nonionic detergent NP-40 (26). Such detergents were previously shown to inhibit PI-3 kinase activity and enhance PI-4 kinase activity (24). Fig. 1 shows the effect of NP-40 on PI-3 kinase activity in

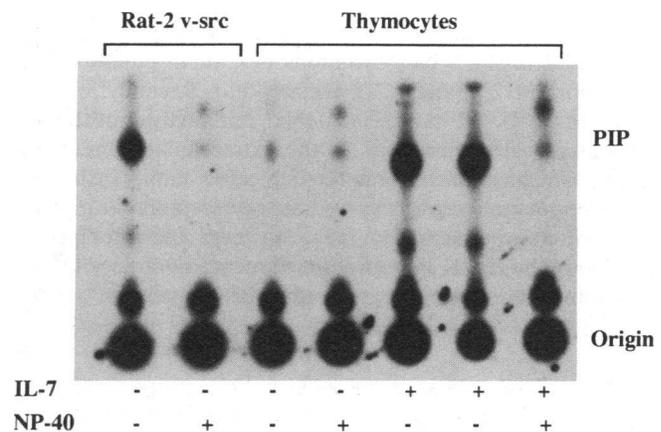


Figure 1. Phosphorylation of PI by phosphotyrosine immunoprecipitates. Thymocytes were stimulated for 15 min with rhIL-7, lysed, immunoprecipitated with anti-phosphotyrosine antibody, and the PI kinase activity determined by measuring the incorporation of γ [^{32}P]-ATP into PI. Fig. 1 shows the separation by TLC of the [^{32}P]PIP reaction products, obtained in the absence (-) or in the presence (+) of 1% NP-40, in immunoprecipitates of v-src-transformed rat 2 fibroblasts cells. Immunoprecipitates of rhIL-7-stimulated or unstimulated human thymocytes showing [^{32}P]PIP reaction products in the absence (-) or in the presence (+) of 1% NP-40 are shown. The position of the migration of PIP and the origin of chromatography are indicated. The data represent one of six experiments with similar results.

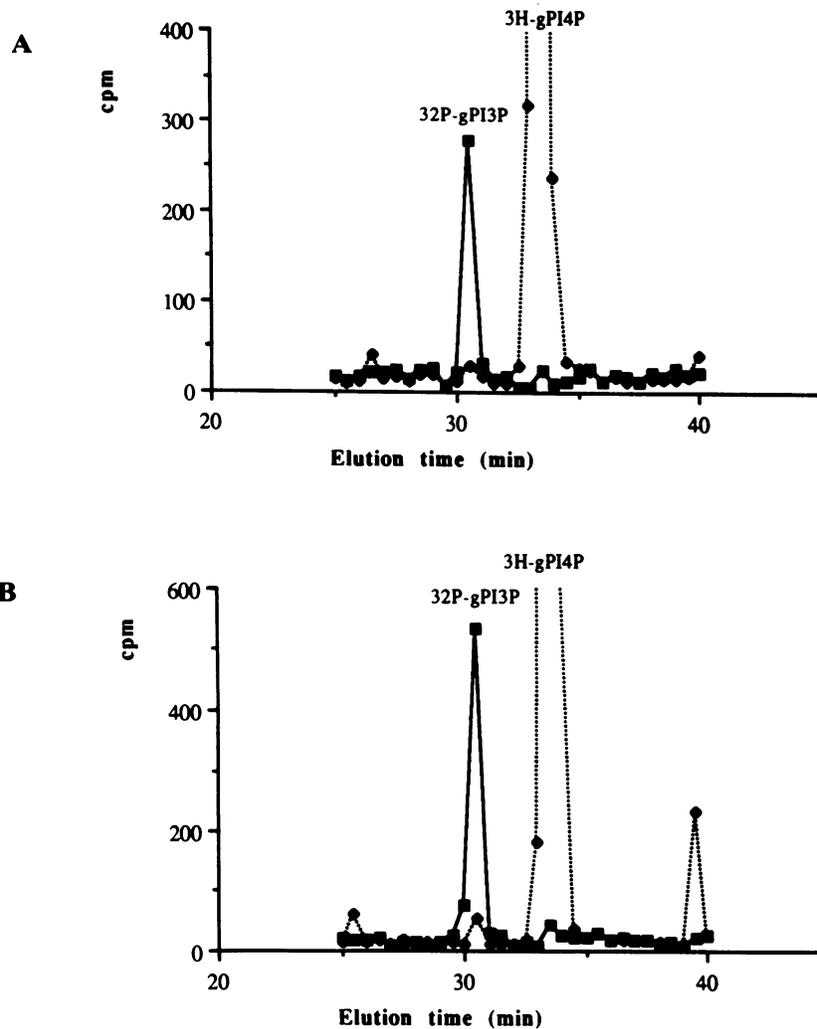


Figure 2. HPLC identification of PI-3 kinase reaction products. The PI-3 kinase products detected in v-src-transformed rat-2 fibroblast cells (A) or in thymocytes stimulated with rhIL-7 (B) were purified from TLC, (non-NP-40-treated samples), deacylated, and analyzed by HPLC. The solid line indicates the elution position (30.5 min) of the glycerol-inositol 3 phosphate form of PI3P. The elution position (33.5 min) of the deacylated [^3H]gPI4P a co-injected standard is indicated by the dotted line. Similar results were obtained in five additional experiments under identical conditions.

rhIL-7-stimulated thymocytes. At 1% NP-40, the PI kinase reaction was completely abolished. Similar concentrations of NP-40 also inhibited PI-3 kinase activity in IL-2-treated CTLL-2 cells (18) and in other receptor systems (26). Direct detection of PI3P in the kinase reaction products was performed by HPLC analysis. The lipid was retrieved from the

TLC plate, deacylated to yield ^{32}P -labeled glycerol-phosphate products gPI3P and gPI4P, which were then separated on an anion exchange column (15). [^{32}P]gPI3P eluted at 30.5 min whereas the coinjected [^3H]gPI4P standard eluted at 33.5 min.

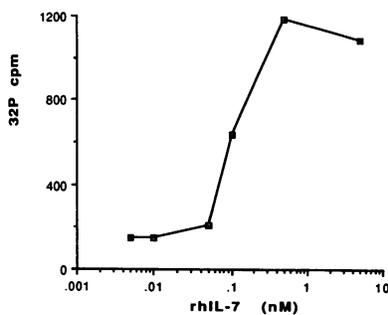


Figure 3. PI-3 kinase activity in thymocytes stimulated with various concentrations of rhIL-7. Thymocytes (2×10^7 cells) were incubated for 15 min at 37°C with concentrations of rhIL-7 as indicated. The cells were lysed and the phosphotyrosine containing proteins immunoprecipitated. Relative PI kinase activity was measured in the immunoprecipitates by the quantification of the [^{32}P]PIP reaction product separated on TLC. The data represent one of five different experiments with similar results.

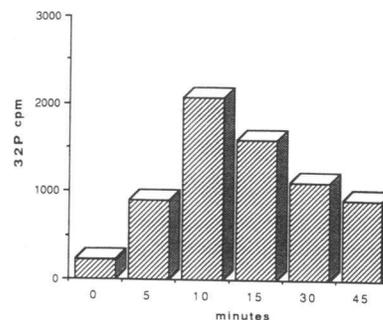


Figure 4. Time course of PI-3 kinase activity in thymocytes stimulated with rhIL-7. Thymocytes (2×10^7 cells) were stimulated with 30 ng (1.7 nM) rhIL-7 for periods of time as indicated and subsequently lysed for 15 min at 4°C . The phosphotyrosine containing proteins were immunoprecipitated and the PI kinase activity measured by the incorporation of ^{32}P into PIP in the absence of 1% NP-40. ^{32}P incorporated into PIP was quantitated by scraping the radio-labeled spots from TLC and counting on a β -counter. The data represent one of three different experiments with similar results.

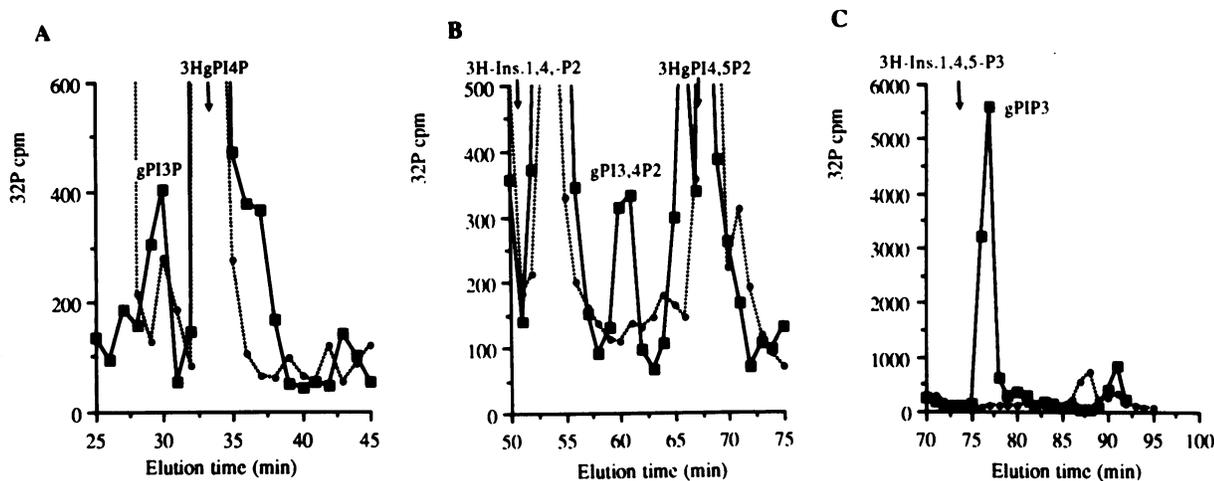


Figure 5. HPLC analysis of polyphosphoinositides from intact thymocytes. Thymocytes were metabolically labeled with $^{32}\text{PO}_4^{3-}$ for 3 h before stimulation for 15 min with rhIL-7. The phospholipids were extracted, deacylated, separated on HPLC, and identified by comparison with the elution properties of [^3H]gPI4P, [^3H]gPI4,5P₂, [^3H]inositol 1,4 bisphosphate, and [^3H]inositol 1,4,5 trisphosphate standards. The dotted line represents phospholipids from unstimulated cells, and the solid line represents phospholipids from rhIL-7-stimulated cells. The arrows indicate the elution positions of [^3H]gPI4P, [^3H]gPI4,5P₂, [^3H]Ins 1,4P₂, and [^3H]Ins 1,4,5P₃ co-injected with the samples. *A* shows the elution profile of gPI3P and gPI4P, *B* shows the elution profile of gPI3,4P₂, [^3H]gPI4,5P₂, and [^3H]Ins 1,4P₂, and *C* shows the elution profile of [^3H]Ins 1,4,5P₃ and gPI3,4,5P₃ (PIP₃). The data represent one of six different experiments with identical results.

As shown in Fig. 2, the major deacylated product in the rhIL-7-treated sample comigrated with gPI3P. Maximal PI-3 kinase activity was achieved by incubating thymocytes with 0.5–1 nM of rhIL-7 as shown by the dose-dependent curve in Fig. 3. Using the optimal concentration of rhIL-7 between 0.5 and 1 nM, PI-3 kinase activity was detected within 5 min of stimulation with maximum activity after 10 min in non-NP-40-treated samples (Fig. 4). NP-40-treated samples consistently showed no significant increase in counts over control (control was 55 cpm and the mean \pm SD for all time points was 61 ± 6). The results, thus, show that ligation of the IL-7R in thymocytes is associated with increased PI-3 kinase activity.

To determine whether stimulation of the IL-7R in thymocytes is activating PI-3 kinase, the accumulation of PI-3 kinase products in intact thymocytes (in vivo) was analyzed. Thymocytes were metabolically labeled with $^{32}\text{PO}_4^{3-}$ and the polyphosphoinositides analyzed by HPLC. Fig. 5 shows a significant increase in the D-3 phosphorylated polyphosphoinositides in rhIL-7-stimulated thymocytes compared with the unstimulated controls, thus confirming that the ligation of the IL-7R in thymocytes activates PI-3 kinase.

Discussion

In this study we demonstrated that phosphotyrosine immunoprecipitates obtained from IL-7-stimulated human thymocytes contained PI-3 kinase activity. This recently discovered phosphatidylinositol kinase phosphorylates polyphosphoinositides at the D-3 position of the inositol ring (15). The function of such inositol lipids, which are phosphorylated at the D-3 position, remains unknown but they do not appear to be substrates for phospholipase C (21, 27) and therefore represent a distinct pathway unrelated to the classical PLC-specific PI pathway.

PI-3 kinase was previously found to coimmunoprecipitate with PDGF (16), colony-stimulating factor 1 (27), insulin re-

ceptors (17), and more recently with the IL-2R (18) in ligand-stimulated cells. Important studies directly linked PI-3 kinase activity with transformation by several oncogene products (21–23).

To determine whether ligation of IL-7R is associated with PI-3 kinase activation, we examined whether antiphosphotyrosine immunoprecipitates obtained from human thymocytes possess PI-3 kinase activity. PI-3 kinase activity was determined by using an in vitro assay that measures the incorporation of ^{32}P in PI to yield [^{32}P]PI3P, the identity of which was confirmed by HPLC analysis (Fig. 2). A marked increase in PI-3 kinase activity was detected in rhIL-7-stimulated thymocytes, a response that was both time and dose dependent (Figs. 3 and 4). IL-7R-associated PI-3 kinase activity was distinguished from PI-4 kinase activity by the addition of nonionic detergent such as NP-40. This detergent was previously shown preferentially to inhibit PI-3 kinase but not PI-4 kinase activity (26). The identification of IL-7-dependent PI-3 kinase activity was also directly confirmed by detection of an increase in deacylated D-3 phosphorylated polyphosphoinositides from intact thymocytes by HPLC (Fig. 5).

These results indicate that rhIL-7-stimulated thymocytes, but not resting thymocytes, contain activated PI-3 kinase.

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