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IN VITRO TYROSINE PHOSPHORYLATION OF PLC-γ1 AND PLC-γ2 BY SRC-FAMILY PROTEIN TYROSINE KINASES

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SUMMARY: The phosphorylation of purified phospholipase C-γ1 (PLC-γ1) and PLC-γ2 by src-family-protein tyrosine kinases (PTKs) $P56^{lck}$, $p53/56^{lyn}$, $p59^{hck}$, $p59^{fyn}$, and $p60^{src}$ was studied *in vitro*. All five PTKs phosphorylated PLC-γ1 and PLC-γ2, suggesting that both PLC-γ isozymes can be phosphorylated in cells by any of the src-family PTKs in response to the activation of cell surface receptors. Comparison of the *in vitro* phosphorylation rates revealed no distinct specificity between PLC-γ1 and PLC-γ2, or between the five PTKs.
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The γ-type isozymes of phospholipase C (PLC), PLC-γ1 and PLC-γ2, are activated as a result of phosphorylation by protein tyrosine kinases (PTKs) (1,2). Phosphorylation is catalyzed by either growth factor receptors that possess ligand-activated cytoplasmic PTK domains or by unidentified nonreceptor PTKs linked to a variety of cell surface receptors (1,2). The activated PLC-γ isozymes catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two second messenger molecules: inositol 1,4,5-trisphosphate and diacylglycerol (3,4).

Treatment of several cell types with peptide growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or nerve growth factor (NGF), elicits a rapid phosphorylation of PLC-γ1 on tyrosine residues and a concomitant increase in PIP_2 hydrolysis (5-9). The major sites of PLC-γ1 phosphorylated in cells in response to the binding of different growth factors appear to be identical and are Tyr-771, Tyr-783, and Tyr-1254 (10). Purified receptors for EGF, PDGF, and NGF also phosphorylate the same tyrosine residues suggesting that tyrosine phosphorylation of PLC-γ1 is mediated directly by the activated receptor PTKs (8-11). In

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contrast to EGF, PDGF, FGF, and NGF, treatment of cells with colony-stimulating factor-1 (CSF-1) or insulin does not elicit the tyrosine phosphorylation of PLC-γ1 (12,13). Furthermore, PLC-γ1 is resistant to phosphorylation by purified insulin receptor (13). Thus, despite the structural similarities between the PTK domains of growth factor receptors, activity of these receptors toward PLC-γ1 does not appear to be universal.

Stimulation of leukocyte surface receptors, such as the T cell antigen receptor (TCR) (14-16), membrane immunoglobulin M (mIgM) in B cells (17-20), the high-affinity IgE receptor ($Fc_{\epsilon}RI$) in mast cells and basophils (21), and IgG receptors ($Fc_{\gamma}RI$, $Fc_{\gamma}RII$, and $Fc_{\gamma}RIII$) in monocytic and natural killer cells (22-25), rapidly induces tyrosine phosphorylation of PLC-γ1 and PLC-γ2, despite the fact that none of the components of these leukocyte receptors is a PTK. The sites of tyrosine phosphorylation in PLC-γ1 in activated T cells are the same as those phosphorylated in cells treated with PDGF or EGF (14). Evidence suggests that src-family PTKs are responsible for the phosphorylation of multiple cellular proteins in response to the stimulation of a variety of membrane receptors in hematopoietic cells. To date, eight src-family PTKs have been identified: the products of src, yes, fgr, lck, fyn, hck, lyn, and blk genes. These src-family PTKs are widely expressed in cells of hematopoietic origin, with most cells expressing more than one of the family members. The TCR was shown to be physically associated with $p59^{fyn}$ (27). Evidence also suggests that multiple members of the src-family PTKs can potentially interact with the same receptor within any one cell type. Thus, $p53/56^{lyn}$, $p59^{fyn}$, $p56^{lck}$, and $p56^{blk}$ coimmunoprecipitate with mIgM in B cells (28-30), and $p56^{lyn}$, $p60^{src}$, and $p62^{yes}$ either coimmunoprecipitate with $Fc_{\epsilon}RI$ or are activated after cross-linking of $Fc_{\epsilon}RI$ in basophils and mast cells (31). It is conceivable, therefore, that multiple PTKs activated by the same receptor exhibit differential substrate specificities. However, there is no data directly showing that any of the src-family PTKs can phosphorylate PLC-γ isozymes.

We have studied the phosphorylation of purified PLC-γ isozymes by various members of the src-family PTKs. Our experiments were designed to address the questions: (i) Do some PTKs exhibit higher activity toward PLC-γ isozymes than other PTKs? (ii) Are PLC-γ1 and PLC-γ2 phosphorylated at different rates by particular PTKs?

MATERIALS AND METHODS

Cells. Jurkat cells (human T cell leukemia cell line), Ramos cells (human B cell lymphoma cell line), and U937 cells (human monocytic cell line) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum. Src B cells, NIH 3T3 cells overexpressing $p60^{src}$, were provided by Dr. J. Bolen (Bristol Myers-Squibb, Princeton, NJ) and maintained in DMEM medium supplemented with 10% fetal bovine serum.

Antibodies and enzymes. All polyclonal antisera directed against unique sequences of each *src*-family PTK were kindly provided by Dr. J. Bolen (32). The sources of $p56^{lck}$, $p53/56^{lyn}$, $p59^{hck}$, and $p60^{src}$ were immunoprecipitates obtained with the corresonding antisera from Jurkat, Ramos, U937, and Src B cells, respectively. The source of $p59^{fyn}$ was the corresponding immunoprecipitate from lymphocyte lysates obtained from LPR mice, which overexpress $p59^{fyn}$ (33). Enolase was purchased from Sigma. PLC-γ1 was purified from bovine brain as described (34), and PLC-γ2 was purified by sequential chromatograph, DEAE, and phenyl HPLC columns from HeLa cells infected with vaccinia virus containing a PLC-γ2 construct (Lee, C.W. and Rhee, S.G., unpublished data). $p56^{lck}$ purified from a vaculovirus expression system was kindly provided by Dr. Julian D. Watts (Biomedical Research Centre, University of British Columbia, Vancouver, Canada) (25).

Immunoprecipitation and *in vitro* kinase assay. Cells were harvested and solubilized in 1% Triton X-100 lysis buffer (1% Triton X-100, 10% glycerol, 50 mM NaF, 0.5 mM phenylmethylsulfonyl fluoride, 1 MM Na_3VO_4, 1 mM EDTA, and 10 μg/ml leupeptin) for 30 min on ice. Postnuclear supernatants were precleared with protein A-agarose (Pierce) prior to immunoprecipitation. The immunoprecipitates were washed twice with lysis buffer without EDTA, once with PBS, and once with water. Immune-complex kinase assays were performed by incubation of washed immunoprecipitates with exogenous substrates [PLC-γ1 (1 μg), PLC-γ2 (1 μg), and enolase (2.5 μg)] in 30 μl of kinase buffer (20 mM Hepes, pH 7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 10 μM ATP, 15 μCi of $[\gamma\text{-}^{32}P]$ATP) for various times at 25°C. The reaction was stopped by addition of Laemmli sample buffer. Reaction mixtures were divided into two equal portions: one portion was subjected to 6% SDS-PAGE to resolve PLC-γ1 and PLC-γ2; and the other portion was subjected to 10% SDS-PAGE to resolve enolase. After electrophoresis, the gels were dried and exposed to a PhosphoImager screen (Molecular Dynamics) for 11 hr. The radioactivity present in PLC-γ1, PLC-γ2, and enolase was quantitated by PhosphoImager.

RESULTS AND DISCUSSION

Because immunoprecipitates obtained with antisera specific to the amino-terminal region of each PTK were used as the source of kinase, comparison of specific activities between different PTKs was not possible. Therefore, enolase, a well-established substrate of src-family PTKs, was added to the assay mixture as an internal standard. To determine whether antibodies bound to the PTKs affected the rate of phosphorylation, we compared the time courses of phosphorylation by $p56^{lck}$ purified by conventional column chromatography (not by immunoprecipitation) with those obtained with immunoprecipitated $p56^{lck}$. Antibodies bound to the amino-terminal region of $p56^{lck}$ did not affect the rates of phosphorylation of PLC-γ1 and PLC-γ2 relative to that of enolase (Fig. 1), suggesting that either the presence of antibodies does not change the catalytic activity of $p56^{lck}$ or that the antibodies influence the reactivities toward PLC-γ1, PLC-γ2, and enolase to the same extent.

The overall amino acid sequence identity between PLC-γ1 and PLC-γ2 is 50.2% (1,2). Two residues, Tyr-753 and Tyr-759, have been identified as phosphorylation sites in PLC-γ2 (12). The sequences surrounding Tyr-753 and Tyr-759 in PLC-γ2 are similar to those surrounding Tyr-771 and Tyr-783 in PLC-γ1. However, PLC-γ2 does not contain a

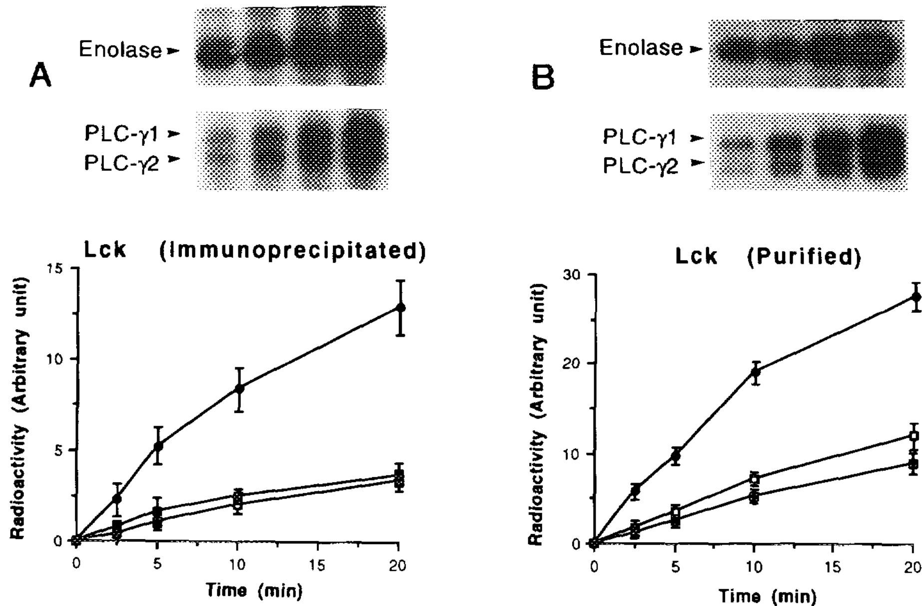

Figure 1. Comparison of the phosphorylation of a mixture of PLC-γ1 (□), PLC-γ2 (■), and enolase (●) by immunoprecipitated p56^{lck} and purified p56^{lck}. Either immunoprecipitated (A) or purified (B) p56^{lck} was incubated with 1 μg each of PLC-γ1 and PLC-γ2, and 2.5 μg of enolase, in 30 μl of kinase buffer. At the indicated times, the reaction mixtures were separated by SDS-PAGE. The gels were exposed to a PhosphoImager screen to yield the autoradiograms shown in the insets. The extent of phosphorylation was measured and expressed in arbitrary units as described in "Materials and Methods". The results are plotted as means ± standard deviation.

tyrosine residue equivalent to Tyr-1254 of PLC-γ1. PLC-γ1 and PLC-γ2 can be separated by SDS-PAGE and exhibit apparent molecular masses of 145 and 138 kDa, respectively. To obviate experimental variability arising from the handling of small quantities of proteins, the substrates were added to all of the phosphorylation assay mixtures from a stock solution containing equal amounts of PLC-γ1 and PLC-γ2.

Each immunoprecipitated PTK, except p60^{src}, phosphorylated PLC-γ1 and PLC-γ2 at similar rates (Figs. 1 and 2); the rate of phosphorylation of PLC-γ1 by p60^{src} was approximately twice that of PLC-γ2. In addition, the phosphorylation rates for PLC-γ1 and PLC-γ2 relative to that for enolase (except the rate of phosphorylation of PLC-γ2 by p60^{src}) were similar for all PTKs. Overall, these results suggest little specificity among src-family PTKs with regard to phosphorylation of PLC-γ isozymes *in vitro*; in contrast to the partial specificity observed with the growth factor receptor PTKs, of which PDGF, EGF, FGF, and NGF receptors phosphorylate PLC-γ1, whereas insulin and CSF-1 receptors fail to do so (5-9,12,13). Little is known about the phosphorylation of PLC-γ2 by growth factor receptor PTKs, except that PLC-γ2 is mainly found in cells of hematopoietic origin (although PLC-γ2

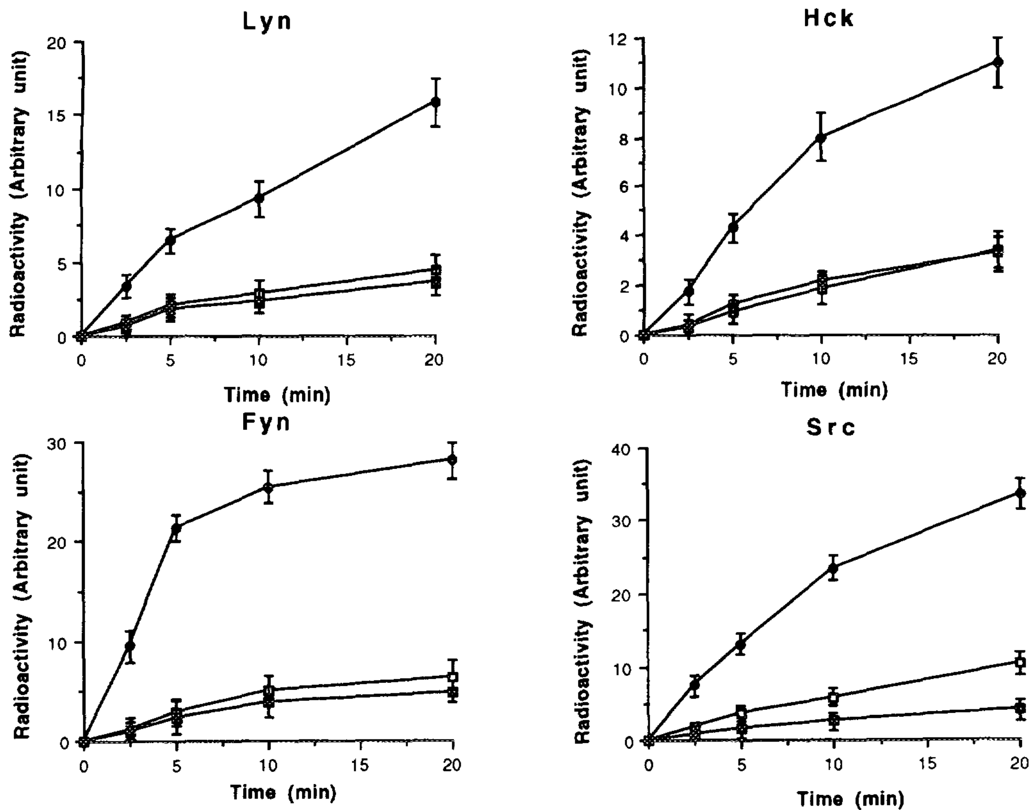

Figure 2. Phosphorylation of PLC-γ1(□), PLC-γ2 (■), and enolase (●) by immunoprecipitated $p56^{lyn}$, $p59^{hck}$, $p59^{fyn}$, and $p60^{src}$. Experimental details are as in Fig. 1.

was first purified from bovine brain) and becomes phosphorylated in response to PDGF when expressed from cDNA in cultured cells (36).

It should be emphasized, however, that in activated cells, both PTKs and PLC-γ isozymes are likely to be present in complexes with other cellular proteins: Antibodies to PLC-γ isozymes coprecipitate several cellular proteins (5,6); the src-family PTKs associate with various receptors and other proteins of unknown function (27,28-31,37). Therefore, specificity of kinase activity may be conferred by the associated proteins in in intact cells.

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