Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 424–428, January 1990
Biochemistry

Selectivity of phospholipase C phosphorylation by the epidermal growth factor receptor, the insulin receptor, and their cytoplasmic domains

(tyrosine kinases/substrate selectivity)

SHUNZO NISHIBE*, MATTHEW I. WAHL*, PHILIP B. WEDEGAERTNER†, JAE JIM KIM‡, SUE GOO RHEE‡, AND GRAHAM CARPENTER*§

Departments of *Biochemistry and §Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-0146; †Department of Chemistry, University of California, San Diego, La Jolla, CA 92093; and ‡Laboratory of Biochemistry, National Heart, Lung and Blood Institute, Bethesda, MD 20892

Communicated by Stanley Cohen, October 20, 1989

ABSTRACT **Phosphatidylinositol-specific phospholipase C isozyme γ (PLC-γ, M_r 145,000) is an excellent substrate for the epidermal growth factor (EGF) receptor both *in vivo* and *in vitro*. PLC-β-1, another PLC isozyme, is a poor substrate for the EGF receptor. We examined the relative phosphorylation of PLC-γ and PLC-β-1 by the 170-kDa native EGF receptor molecule, the 66-kDa cytoplasmic kinase domain of the EGF receptor (Arg^{647}–Ala^{1186}), the $\alpha_2\beta_2$ native insulin receptor, and the 48-kDa cytoplasmic kinase domain of the insulin receptor β subunit (Gly^{947}–Ser^{1343}). Similar to the intact EGF receptor, the cytoplasmic kinase domain of the EGF receptor preferentially phosphorylated PLC-γ. High-performance liquid chromatographic comparison of tryptic phosphopeptides from PLC-γ phosphorylated by both forms of the EGF receptor kinase indicated similar patterns of multiple tyrosine phosphorylations. These results imply that substrate selectivity, at least in terms of PLC isozymes, is independent of the extracellular ligand-binding and membrane anchor domains of the EGF receptor. In comparison, neither the intact insulin receptor nor the β-chain kinase domain was able to phosphorylate PLC-γ to a significant extent. Also, insulin failed to stimulate the phosphorylation of PLC-γ in NIH 3T3/HIR cells, which overexpress the human insulin receptor. Thus PLC-γ is not a phosphorylation substrate for the insulin receptor *in vitro* or in the intact cell.**

Tyrosine kinase activity is an intrinsic and essential property of many transforming gene products and their cellular homologs, including plasma membrane receptors for several growth factors, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), colony-stimulating factor 1, fibroblast growth factor, insulin, and insulin-like growth factor I. Tyrosine phosphorylation is believed to be crucial for the control of both normal cell growth and cellular transformation. Activation of receptor tyrosine kinase activity after ligand binding is the primary transmembrane signaling event for growth factor regulation of cell proliferation. Furthermore, cells expressing kinase-deficient growth factor receptors have been reported as unresponsive to the appropriate growth factor (reviewed in refs. 1–3). Thus, identification of physiological substrates for receptor tyrosine kinases is important to elucidate the biochemical mechanisms underlying the regulation of cell proliferation.

The EGF receptor and the insulin receptor share sequence homologies in their tyrosine kinase domains (4), and both receptors exhibit a similar, but not identical, substrate specificity *in vitro* (5–8). However, there are differences in diverse cell responses to growth factors that stimulate different tyrosine kinases in the same cell. It is likely that each receptor kinase recognizes a distinct, but overlapping, spectrum of substrate proteins whose phosphorylation may signal a particular cell function. Little is known, however, about the physiological substrates for these receptor kinases. Recently, phosphatidyl-inositol specific phospholipase C (PLC) isozyme γ (PLC-γ, formerly PLC-II, M_r 145,000; ref. 9) was reported to be phosphorylated by the EGF and PDGF receptors *in vivo* and *in vitro* (10–14). PLC plays an important role in hormone signaling systems since it hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate, a mediator of intracellular Ca^{2+} release, and 1,2-diacylglycerol, an activator of protein kinase C. Both of these products have second-messenger functions in various hormone signaling systems.

Since the differences or the similarities in the cellular responses to the various growth factors could reflect *in vivo* substrate selection by the receptor kinases, it is important to determine the structural elements of the receptor kinases and their substrates that influence phosphorylation selectivity. In the present study, we have examined the *in vitro* phosphorylation of PLC isozymes by the intact EGF and insulin receptor kinases, purified from mammalian cells, and by their cytoplasmic domains, purified as recombinant proteins expressed in the baculovirus system (15, 16).

METHODS

Cell Culture. NIH mouse 3T3 cells expressing human insulin receptor (NIH 3T3/HIR cells; ref. 17), generously supplied by J. Whittaker (University of Chicago), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (GIBCO) and gentamicin (Sigma). Cells were subcultured for phosphorylation experiments by seeding in 60-mm dishes (Becton Dickinson) containing DMEM plus 10% calf serum (GIBCO) and were grown to 70–80% confluence. For the insulin receptor isolation, cells were grown to 100% confluence in a 245 mm × 245 mm culture dish (Nunc).

Preparation of Tyrosine Kinases. The EGF receptor was purified from solubilized plasma membrane vesicles (18) of A-431 human epidermoid carcinoma cells by affinity chromatography on wheat germ lectin Sepharose 6MB (Sigma) as described (14). The cytoplasmic kinase domain of the EGF receptor was expressed in Sf9 insect ovary cells with the baculovirus expression system and purified to near homogeneity with a monoclonal anti-phosphotyrosine antibody column (15). The insulin receptor was prepared from Triton X-100-solubilized plasma membrane of NIH 3T3/HIR cells by the same procedures as used for the EGF receptor

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; BIRK, baculovirus insulin receptor kinase; PLC, phosphatidylinositol-specific phospholipase C.

preparation (14). Purified baculovirus-expressed insulin receptor kinase (BIRK), comprising the cytoplasmic domain of the insulin receptor β chain (16), was generously supplied by O. Rosen (Sloan–Kettering Cancer Center).

Protein Kinase Assay. PLC isozymes purified from bovine brain (19) were phosphorylated by the intact EGF receptor (0.2 μg) or the kinase domain of the EGF receptor (0.2 μg) in 25-μl reaction mixtures containing (unless otherwise indicated) 20 mM Hepes (pH 7.4), 25 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1 mM Na_3VO_4, 20 μM [γ-^{32}P]ATP (20 μCi/nmol; 1 μCi = 37 kBq), and 0.5 μg of PLC. In the phosphorylation by the EGF receptor, 0.3 μM EGF isolated from mouse submaxillary glands (20) was preincubated with the EGF receptor for 10 min at 22°C. The insulin receptor (0.2 μg) or BIRK (0.5 μg) was incubated in a reaction mixture (25 μl) containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DL-dithiothreitol, 0.1 mM Na_3VO_4, and 100 μM [γ-^{32}P]ATP (20 μCi/nmol) after preincubation with 0.5 μM bovine insulin (Sigma) for 10 min at 22°C. The reaction was initiated by the addition of [γ-^{32}P]ATP and terminated, after 15 min at 22°C, by the addition of Laemmli buffer (21) and heating for 5 min at 60°C. Previous studies (14) showed that EGF receptor phosphorylation of PLC-γ is ≈80% complete under these conditions. The samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) (21). Phosphoprotein bands were visualized by autoradiography, and incorporation of phosphate was measured by excising the band and assaying the amount of ^{32}P by Cerenkov counting.

Immunoprecipitation of PLC. To obtain the radiolabeled proteins, NIH 3T3/HIR cells were incubated with [^{35}S]methionine/[^{35}S]cysteine (Tran^{35}S-label; ICN) at 50 μCi/ml in DMEM plus 10% calf serum for 20 hr or with [^{32}P]orthophosphate (ICN) at 0.75 mCi/ml in phosphate-free minimum essential medium plus 1% dialyzed calf serum for 2 hr prior to the treatment of insulin. ^{35}S-labeled cells or ^{32}P-labeled cells after insulin (0.5 μg/ml) treatment for 10 min were washed four times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and radiolabeled proteins were extracted with 1 ml of 20 mM Hepes, pH 7.4/100 mM NaCl/1% (vol/vol) Triton X-100/10% (vol/vol) glycerol/1 mM phenylmethylsulfonyl fluoride/0.1 mM Na_3VO_4 containing leupeptin at 10 μg/ml. Immunoprecipitation of PLC-β-1, PLC-γ, PLC-δ, and human insulin receptor was performed as described (10). The immunoprecipitated proteins were analyzed by SDS/7.5% PAGE and visualized by fluorography (^{35}S) or autoradiography (^{32}P).

Tryptic Phosphopeptide Mapping. A slice of SDS gel corresponding to the PLC-γ band was excised and incubated in 0.5 ml of 0.1 M NH_4HCO_3 with 1 μg of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) for 20 hr at 4°C. After collection of the supernatant, the gel slice was incubated again with 1 μg of trypsin for 2 hr at 22°C. The supernatants were combined and the proteolysis was terminated by heating at 70°C for 20 min. The tryptic phosphopeptides were separated by reverse-phase high-performance liquid chromatography (HPLC) on a C_8 column (Microsorb, 5-μm particle size; 4.6 mm × 25 cm; Rainin Instruments, Woburn, MA). The column was equilibrated in 0.1% trifluoroacetic acid, and phosphopeptides were eluted at a flow rate of 0.5 ml/min with an increasing concentration of acetonitrile. The radioactivity in each fraction (0.5 ml) was measured by liquid scintillation counting with ACS aqueous counting scintillant (Amersham).

RESULTS AND DISCUSSION

Phosphorylation of PLC Isozymes by EGF Receptor Kinases. Recently, we demonstrated (14) that while PLC-γ (M_r 145,000) was an efficient phosphorylation substrate for the purified EGF receptor *in vitro*, PLC-β-1 (M_r 150,000, formerly PLC-I) was a poor substrate and PLC-δ (M_r 85,000, formerly PLC-III) was not phosphorylated to any detectable extent. This high selectivity among PLC isozymes for *in vitro* phosphorylation by the EGF receptor is somewhat surprising, as all three PLC isozymes possess typical tyrosine phosphorylation sequences. Little is known about receptor structural requirements that lead to this selective phosphorylation of PLC-γ. To examine whether the cytoplasmic domain of the EGF receptor, lacking the extracellular ligand-binding domain and the transmembrane domain, exhibits the same substrate selectivity, we compared the phosphorylation of PLC isozymes by the intact EGF receptor kinase and the cytoplasmic kinase domain of the EGF receptor. Intact EGF receptor (residues 1–1186) was purified from A-431 cells (14), while the receptor fragment containing the cytoplasmic domain (residues 647–1186) was purified from a baculovirus expression system using cells infected with a virus containing a cDNA encoding this portion of the human EGF receptor (15). The results (Fig. 1) demonstrate that the cytoplasmic kinase domain phosphorylates PLC-γ as well as the intact receptor (lanes 2 and 4) but only weakly phosphorylates PLC-β-1 (lanes 1 and 3). Similar selective phosphorylation was observed when sphingosine, which activates this recombinant kinase (15), was present in the phosphorylation assay. The ratio of PLC-γ phosphorylation to receptor autophosphorylation, calculated from the radioactivity incorporated into both proteins, was similar for the intact receptor (2.3) and the kinase domain (2.5). The ratio of PLC-β-1 phosphorylation to receptor autophosphorylation was 0.1 and 0.6 for the intact receptor and cytoplasmic kinase domain, respectively. Since the cytoplasmic kinase domain exhibited the same selective phosphorylation of PLC-γ as the intact receptor, neither the extracellular domain, containing the ligand binding site, nor the transmembrane domain is required

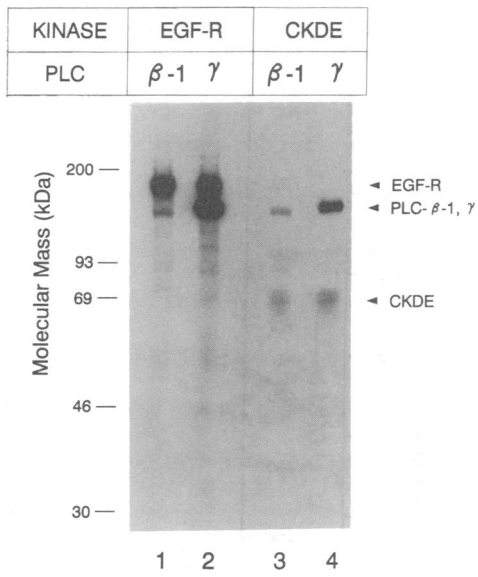

FIG. 1. Phosphorylation of PLC isozymes by the native EGF receptor and its cytoplasmic kinase domain *in vitro*. Five-tenths of a microgram of PLC-β-1 (lane 1) or PLC-γ (lane 2) was incubated with purified EGF receptor (EGF-R, 0.2 μg) in the presence of 0.3 μM EGF. Similarly, 0.5 μg of PLC-β-1 (lane 3) or PLC-γ (lane 4) was incubated with the cytoplasmic kinase domain of the EGF receptor (CKDE, 0.2 μg). The reaction was initiated by the addition of 20 μM [γ-^{32}P]ATP (20 μCi/nmol) and terminated by the addition of Laemmli sample buffer after 15 min at 22°C. The results of Cerenkov counting of PLC phosphorylation and autophosphorylation bands excised from the gel were as follows: lane 1, PLC-β-1 (2048 cpm), EGF receptor (17,436 cpm); lane 2, PLC-γ (39,737 cpm), EGF receptor (15,710 cpm); lane 3, PLC-β-1 (1260 cpm), kinase domain (2261 cpm); lane 4, PLC-γ (6092 cpm), kinase domain (2428 cpm).

for substrate selection by the EGF receptor. This conclusion is in accord with previous studies defining the influence of EGF on kinetic parameters of substrate phosphorylation by the EGF receptor (21). Those results consistently have shown that activation of receptor kinase by EGF binding increases the V_{max} for substrate phosphorylation but has no significant influence on the K_m value.

We used HPLC analysis of PLC-γ tryptic phosphopeptides to further examine whether phosphorylation of PLC-γ by the EGF receptor kinase domain is similar to phosphorylation by the intact receptor. Fig. 2 shows the HPLC elution profiles of phosphopeptides prepared from PLC-γ phosphorylated by either the cytoplasmic kinase domain or the intact EGF receptor. These two elution profiles are very similar, suggesting that the same tyrosine sites are phosphorylated. We conclude, therefore, that the cytoplasmic kinase domain can closely mimic the intact EGF receptor in the phosphorylation of PLC-γ.

Phosphorylation of PLC-γ by Insulin Receptor Kinases. The insulin receptor kinase and the EGF receptor kinase have been reported to phosphorylate certain substrates in a similar manner (5, 6). For example, angiotensin II, gastrin, myosin light chain, casein, histone, tubulin, the progesterone receptor, and lipocortin are substrates for both kinases *in vitro* (7, 8, 22, 23). With the exception of the 35-kDa lipocortin-like protein (24, 25), whose physiological role is uncertain, these proteins are not known to be cellular substrates for either kinase. Therefore, we tested the capability of PLC isozymes to act as substrates for the insulin receptor. When PLC-β-1 and PLC-γ were incubated individually with the purified intact human insulin receptor and [γ-^{32}P]ATP, the levels of tyrosine phosphorylation of the two isozymes were equivalent (Fig. 3*A*, lanes 2 and 3). In this experiment, the ratio of PLC-γ phosphorylation to receptor autophosphorylation was 2.4 for the intact EGF receptor (lane 4) and 0.7 for the intact insulin receptor (lane 3), while the ratio of PLC-β-1 phosphorylation to autophosphorylation of the insulin receptor was 0.7. We obtained similar results when BIRK was used (Fig. 3*B*, lanes 7 and 8). Under the same conditions, both the intact insulin receptor and BIRK phosphorylated tubulin and RR-SRC (data not shown), a synthetic peptide corresponding to the sequence of the tyrosine phosphorylation site in pp60^{src} (26).

Although PLC-γ phosphorylation by the insulin receptor *in vitro* was significantly less than that produced by the EGF receptor, we examined whether PLC-γ phosphorylation could be stimulated by insulin treatment of intact cells. NIH 3T3/HIR cells, which overexpress the transfected human insulin receptor (6×10^6 receptors per cell), were metabolically labeled with [^{35}S]methionine and aliquots of cell extract were immunoprecipitated with antibodies to three distinct PLC isozymes: β-1 (150 kDa), γ (145 kDa), and δ (85 kDa) (27). The results (Fig. 4*A*) indicate that PLC-γ (lane 2) is the most abundant isozyme present in these cells. NIH 3T3/HIR cells were then radiolabeled in medium containing [^{32}P]orthophosphate for 2 hr, treated with insulin for 10 min, and solubilized. PLC-γ and the insulin receptor were immunoprecipitated and phosphoproteins were visualized by autoradiography (Fig. 4*B*). No increase in the phosphorylation of PLC-γ was apparent (lanes 4 and 5), although insulin increased autophosphorylation of the insulin receptor β chain (lanes 6 and 7). Phospho amino acid analysis of PLC-γ from control and insulin-treated cells showed the presence of phosphoserine only (data not shown). These results are consistent with the data shown in Fig. 3 and suggest that PLC-γ is not a good substrate for the insulin receptor either *in vitro* or *in vivo*.

Growth factor-stimulated tyrosine phosphorylation of PLC-γ in intact cells was initially observed by Wahl *et al.* (28) and was correlated with phosphatidylinositol hydrolysis. PDGF and EGF stimulate phosphatidylinositol hydrolysis in transformed and nontransformed cell lines (reviewed in ref. 29) and their receptors selectively phosphorylate PLC-γ *in vivo*. We now show that PLC-γ is not selectively phospho-

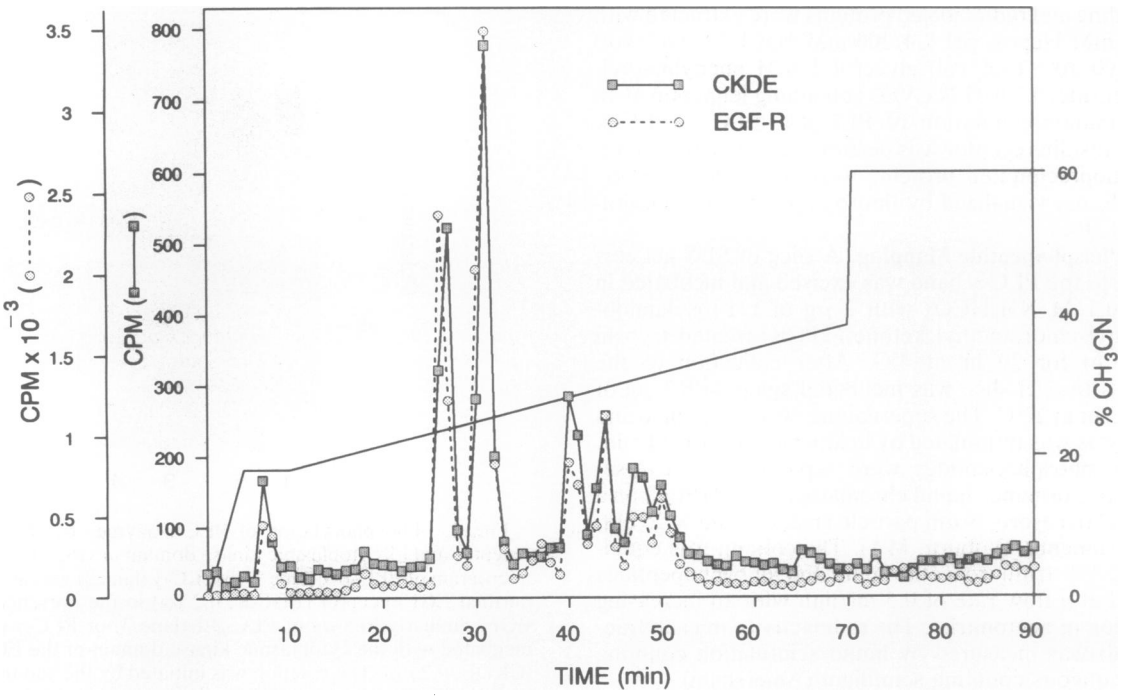

FIG. 2. HPLC analysis of tryptic phosphopeptides from PLC-γ phosphorylated by the EGF receptor (EGF-R) or the cytoplasmic kinase domain of the EGF receptor (CKDE). PLC-γ (0.5 μg) was incubated with either the intact receptor or the kinase domain in the presence of 20 μM [γ-^{32}P]ATP (20 μCi/nmol) at 22°C for 15 min. Reactions were terminated with Laemmli buffer and SDS/PAGE was performed. The gel slice corresponding to PLC-γ was excised and digested with trypsin (see *Methods*). Tryptic phosphopeptides were analyzed on a C_8 reverse-phase column. Phosphopeptides were eluted at a flow rate of 0.5 ml/min with the indicated acetonitrile gradient (—). The ^{32}P-labeled phosphopeptides were detected by liquid scintillation counting.

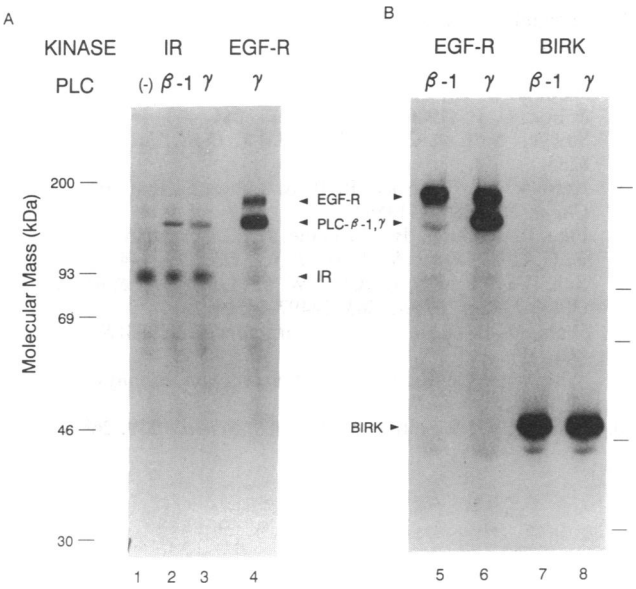

FIG. 3. Comparative phosphorylation of PLC isozymes by the EGF receptor and the insulin receptor. The intact insulin receptor (IR, 0.2 μg) (*A*) or the β-chain cytoplasmic domain of the insulin receptor (BIRK, 0.5 μg) (*B*) was compared with the EGF receptor (EGF-R, 0.2 μg) for the ability to phosphorylate PLC-β-1 or PLC-γ (0.5 μg) when incubated in the presence of 100 μM [γ-^{32}P]ATP (20 μCi/nmol) at 22°C for 15 min. The reaction conditions for insulin receptor kinase assay (see *Methods*) were used for PLC isozyme phosphorylation by the EGF receptor. SDS/7.5% PAGE and autoradiography were carried out to detect phosphorylated proteins. The exposure time was 1 hr at −70°C for *A* and 2 hr at −70°C for *B*. The position of the insulin receptor β subunit in the autoradiogram is indicated by IR. The results of Cerenkov counting of PLC and insulin receptor bands excised from the gel were as follows: lane 1, insulin receptor (2380 cpm); lane 2, PLC-β-1 (1760 cpm), insulin receptor (2130 cpm); lane 3, PLC-γ (1650 cpm), insulin receptor (2220 cpm); lane 4, PLC-γ (7310 cpm), EGF receptor (3024 cpm); lane 5, PLC-β-1 (520 cpm), EGF receptor (6920 cpm); lane 6, PLC-γ (10,330 cpm), EGF receptor (6890 cpm); lane 7, PLC-β-1 (not detectable), BIRK (8240 cpm); lane 8, PLC-γ (not detectable), BIRK (8290 cpm).

rylated by the insulin receptor. While the insulin receptor is able to activate a phospholipase for the hydrolysis of glycosyl-phosphatidylinositol-anchored proteins on the cell surface (30), this activity does not liberate inositol 1,4,5-trisphosphate (30). Furthermore, there are no confirmed reports of significant insulin-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate. Therefore, the failure of the insulin receptor to phosphorylate PLC-γ is consistent with current knowledge of insulin mediators. These data also accentuate the specificity of PLC-γ phosphorylation by the EGF receptor, since a related tyrosine kinase—i.e., the insulin receptor, which does phosphorylate other substrates in common with the EGF receptor—is unable to phosphorylate this PLC isozyme selectively. Our findings demonstrate the most discriminating substrate selectivity observed in this protein kinase family to date and strengthen the hypothesis that PLC-γ is a biologically relevant substrate for the EGF and PDGF receptors.

We thank Dr. Ora Rosen (Sloan–Kettering Cancer Center) for the generous gift of BIRK and Dr. Jonathan Whittaker for the kind gifts of NIH 3T3/HIR cells and the insulin receptor antibody. We thank Kelly Adair for technical assistance and Sue Carpenter for preparation of the manuscript. This work was supported by Research Grant CA43720 from the National Cancer Institute and Training Grants GM07347 and DK07563 from the National Institutes of Health.

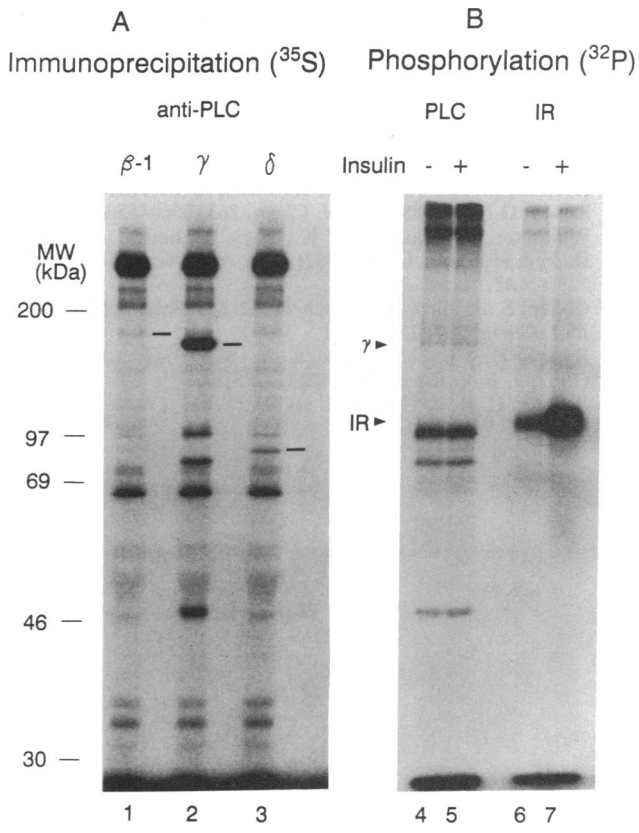

FIG. 4. Influence of insulin on phosphorylation of PLC-γ in NIH 3T3/HIR cells. NIH 3T3/HIR cells were radiolabeled with [^{35}S]-methionine (*A*) or [^{32}P]orthophosphate (*B*). (*A*) PLC-β-1 (lane 1), PLC-γ (lane 2), or PLC-δ (lane 3) was immunoprecipitated from ^{35}S-radiolabeled cell extracts and analyzed by SDS/7.5% PAGE. The gel was treated with Enlightening (NEN) and then exposed to Kodak X-AR film for 72 hr at −70°C. The position of the insulin receptor β subunit in the autoradiogram is indicated by IR. (*B*) PLC-γ (lanes 4 and 5) or the insulin receptor (IR, lanes 6 and 7) was immunoprecipitated from ^{32}P-labeled extracts of NIH 3T3/HIR cells treated without (lanes 4 and 6) or with (lanes 5 and 7) insulin (0.5 μg/ml) for 10 min. Immunoprecipitated proteins were analyzed by SDS/7.5% PAGE. Autoradiographic exposure time was 3 hr at −70°C.

1. Kahn, C. R. & White, M. F. (1988) *J. Clin. Invest.* **82,** 1151–1156.
2. Blackshear, P. J., Nairn, A. C. & Kuo, J. F. (1988) *FASEB J.* **2,** 2957–2969.
3. Carpenter, G. & Wahl, M. I. (1990) in *Peptide Growth Factors and Their Receptors: Handbook of Experimental Pharmacology*, eds. Roberts, A. & Spoun, M. (Springer, Heidelberg, FRG), Vol. 95, pp. 69–171.
4. Hanks, S. K., Quinn, A. M. & Hunter, T. (1988) *Science* **241,** 42–52.
5. Pike, L. J., Kuenzel, E. A., Casnellie, J. E. & Krebs, E. G. (1984) *J. Biol. Chem.* **259,** 9913–9921.
6. Akiyama, T., Kadowaki, T., Nishida, E., Kadooka, T., Ogawara, H., Fukami, Y., Sakai, H., Takaku, F. & Kasuga, M. (1986) *J. Biol. Chem.* **261,** 14797–14803.
7. Fava, R. A. & Cohen, S. (1986) *J. Biol. Chem.* **259,** 2636–2645.
8. Karasik, A., Pepinsky, R. B., Shoelson, S. E. & Kahn, C. R. (1988) *J. Biol. Chem.* **263,** 11862–11867.
9. Rhee, S. G., Suh, P.-G., Ryu, S.-H. & Lee, S. Y. (1989) *Science* **244,** 546–550.
10. Wahl, M. I., Nishibe, S., Suh, P.-G., Rhee, S. G. & Carpenter, G. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1568–1572.
11. Wahl, M. I., Olashaw, N. E., Nishibe, S., Rhee, S. G., Pledger, W. J. & Carpenter, G. (1989) *Mol. Cell. Biol.* **9,** 2934–2943.
12. Meisenhelder, J., Suh, P.-G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57,** 1109–1122.

13. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. & Schlessinger, J. (1989) *Cell* **57,** 1101–1107.
14. Nishibe, S., Wahl, M. I., Rhee, S. G. & Carpenter, G. (1989) *J. Biol. Chem.* **264,** 10335–10338.
15. Wedegaertner, P. B. & Gill, G. N. (1989) *J. Biol. Chem.* **264,** 11346–11353.
16. Herrera, R., Lebwohl, D., de Herreros, A. G., Kallen, R. G. & Rosen, O. M. (1988) *J. Biol. Chem.* **263,** 5560–5568.
17. Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F. & Hoffman, C. A. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 5237–5241.
18. Cohen, S., Ushiro, H., Stoscheck, C. & Chinkers, M. (1982) *J. Biol. Chem.* **257,** 1523–1531.
19. Ryu, S.-H., Cho, K. S., Lee, K.-Y., Suh, P.-G. & Rhee, S. G. (1987) *J. Biol. Chem.* **262,** 12511–12518.
20. Savage, C. R. & Cohen, S. (1972) *J. Biol. Chem.* **247,** 7609–7611.
21. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
22. Gammeltoft, S. & Van Obberghen, E. (1986) *Biochem. J.* **235,** 1–11.
23. Woo, D. D. L., Fay, S. P., Griest, R., Coty, W., Goldfine, I. & Fox, C. F. (1986) *J. Biol. Chem.* **261,** 460–467.
24. Sawyer, S. T. & Cohen, S. (1985) *J. Biol. Chem.* **260,** 8233–8236.
25. Karasik, A., Pepinsky, R. B. & Kahm, C. R. (1988) *J. Biol. Chem.* **263,** 18558–18562.
26. Pike, L. J., Gallis, B., Casnellie, J. E., Bornstein, P. & Krebs, E. G. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1443–1447.
27. Suh, P.-G., Ryu, S. H., Choi, W. C., Lee, K.-Y. & Rhee, S. G. (1988) *J. Biol. Chem.* **263,** 14497–14504.
28. Wahl, M. I., Daniel, T. O. & Carpenter, G. (1988) *Science* **241,** 968–970.
29. Whitman, M. & Cantley, L. (1988) *Biochim. Biophys. Acta* **948,** 327–344.
30. Low, M. G. & Saltiel, A. R. (1988) *Science* **239,** 268–275.