Antiphosphotyrosine Recovery of Phospholipase C Activity After EGF Treatment of A-431 Cells

MATTHEW I. WAHL, THOMAS O. DANIEL, GRAHAM CARPENTER*

A tenfold increase in phospholipase C activity specific for phosphatidylinositol 4,5-bisphosphate (PIP_2) was immunopurified from extracts of A-431 epidermoid carcinoma cells stimulated with epidermal growth factor. This finding suggests a biochemical link between growth factor–stimulated tyrosine kinase activity and PIP_2 hydrolysis.

EPIDERMAL GROWTH FACTOR (EGF) is a small polypeptide hormone that modulates proliferation and metabolism in a wide variety of cell types (*1*). However, the mechanisms by which EGF or other growth factors modulate cellular function are not clearly understood. Although a primary biochemical response following EGF binding is stimulation of the intrinsic tyrosine kinase activity of the EGF receptor (*2*), we (*3*) and others (*4*) demonstrated that EGF stimulates the rapid formation of inositol 1,4,5-trisphosphate (IP_3) in certain cell types. Also, other growth factors also bind to receptors that have intrinsic tyrosine kinase activity (*5*) and rapidly produce a number of biochemical changes in cells, such as the formation of IP_3 (an intracellular second messenger for the liberation of stored Ca^{2+}) and diacylglycerol (an endogenous activator of protein kinase C) by the stimulation of hydrolysis of PIP_2 (*6*). A large number of oncogene products have endogenous tyrosine kinase activity (*7*) and some are reported to affect phosphoinositide metabolism (*8*). However, physiologically important substrates for tyrosine kinases, although central to the regulation of cell growth and transformation, have not yet been identified.

We showed earlier (*3*) that EGF treatment of A-431 cells initiates the rapid formation of IP_3 in the absence of extracellular Ca^{2+} (within 1 minute of EGF addition). After approximately 1 minute of EGF stimulation, continued accumulation of inositol phosphates is markedly stimulated by extracellular Ca^{2+} (*3*). Our data from [^{3}H]inositol-labeled intact cells suggest that EGF stimulation of the receptor tyrosine kinase might initiate PIP_2 hydrolysis through tyrosine phosphorylation of a substrate protein necessary for activation of phospholipase C. We used an immobilized antibody to phosphotyrosine (*9*) to separate proteins phosphorylated on tyrosine from cells treated with or without EGF and then measured phospholipase C activity in vitro.

In the experiment shown in Fig. 1A, we treated A-431 cells with or without EGF for 0 to 60 minutes and then prepared a soluble extract. Sepharose-linked antibody to phosphotyrosine was added to the crude extracts and, following adsorption, the matrix was washed free of nonadsorbed protein. Proteins bound specifically through phosphotyrosine to the matrix were eluted with phenylphosphate, a phosphotyrosine analog. When portions of these eluates from EGF-stimulated and control cells were assayed for phospholipase C activity by using phosphatidyl-[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2) as a substrate, there was a substantial increase in phospholipase C activity in eluates from EGF-treated cells as compared to control cells (Fig. 1A). A tenfold increase in phospholipase C activity was detected within 1 minute of EGF addition to the cells. The strong anion exchange elution profile of water-soluble ^{3}H-labeled compound generated in this assay (Fig. 2) demonstrates that only the 1,4,5-trisphosphate isomer of IP_3 was formed. Separate experiments showed that the release of radioactivity from [^{3}H]PIP_2 during the phospholipase C assay was linear for 20 minutes and that the activity detected was proportional to the amount of eluate (5 to 20 μl) assayed. Without phosphotyrosine immunoisolation, no difference in phospholipase C activity was detected in extracts from control and EGF-treated cells.

The correlation of the EGF-increased phospholipase C activity measured in vitro (Fig. 1A) to that observed in intact cells is demonstrated in Fig. 1B, which shows the time course of EGF-stimulated formation of [^{3}H]inositol trisphosphates in intact A-431 cells prelabeled with [^{3}H]inositol. These results demonstrate that whether IP_3 formation is measured in vivo or in vitro after EGF treatment, the time course for the generation of increased phospholipase C activity is similar.

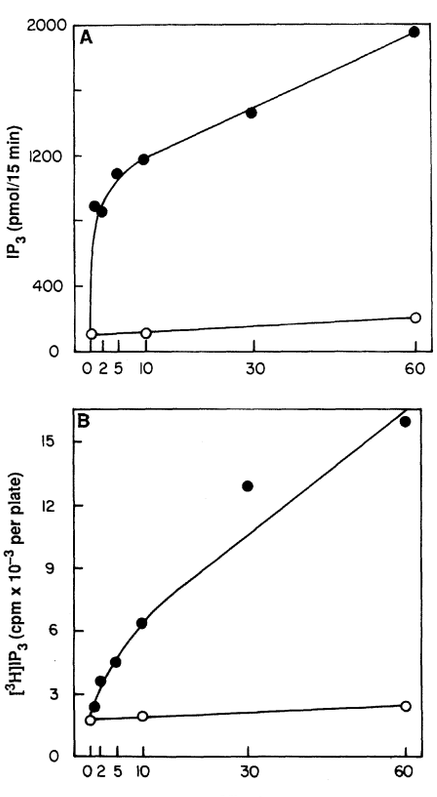

Fig. 1. (**A**) Time course of appearance of phosphotyrosine-immunopurified phospholipase C activity from untreated (○) and EGF-treated (●) A-431 cells. Aliquots (20 μl) of antiphosphotyrosine eluates from cells (*12*) treated without or with EGF (200 ng/ml) for 0 to 60 minutes were added to the phospholipase C reaction mixtures and incubated for 15 minutes at 37°C. The assays were ended by placing the reaction mixtures in an ice-water bath and adding 100 μl of 1% bovine serum albumin followed by 500 μl of 10% trichloroacetic acid. The precipitate was removed by centrifugation, the acidic supernatant was extracted four times with 2 ml of ethyl ether, and the radioactivity present in the extracted samples was determined by scintillation counting with aqueous counting scintillant. The radioactivity present in the acid supernatant of a 20-μl elution buffer–only control reaction mixture (310 cpm) was subtracted from that present in the experimental samples. The difference was converted to picomoles of IP_3 based on the initial substrate specific activity (4450 cpm/nmol). The results are presented as the amount of phospholipase C activity present in the total eluates (200 μl). (**B**) Time course of formation of [^{3}H]inositol trisphosphates in intact A-431 cells treated without (○) or with (●) EGF. A-431 cells (4×10^6 cells per 100-mm plate) in DMEM plus 10% calf serum were labeled for 18 hours with [^{3}H]inositol (2 μCi/ml) and then treated without or with EGF (200 ng/ml) in the presence of LiCl (20 m*M*) for 0 to 60 minutes (*3*). Water-soluble [^{3}H]inositol-containing compounds were recovered from the cells and separated by Dowex anion exchange chromatography (*3*). The [^{3}H]IP_3 fraction also contains [^{3}H]inositol 1,3,4,5-tetrakisphosphate, produced by phosphorylation of [^{3}H]IP_3, and [^{3}H]inositol 1,3,4-trisphosphate (*3*). The data in both (A) and (B) represent the average of duplicate assays that varied by less than 5%.

M. I. Wahl, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232.
T. O. Daniel, Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232.
G. Carpenter, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232.

*To whom correspondence should be addressed.

To evaluate whether the immunoaffinity purification of phospholipase C activity was through specific recognition of phosphotyrosine by the antibody matrix, we performed a control experiment. Crude extracts from EGF-treated cells were adsorbed to the antiphosphotyrosine matrix in separate tubes in the presence of tyrosine, phosphothreonine, phosphoserine, or phosphotyrosine (final concentrations, 1 m*M*). After the matrix was washed to remove nonadsorbed protein, eluates were obtained and assayed for phospholipase C activity. The presence of phosphotyrosine during the adsorption step reduced by 75% the amount of phospholipase C activity subsequently eluted from the matrix. Incubation with phosphothreonine, phosphoserine, or tyrosine did not interfere with the adsorption and elution of phospholipase C activity from the antiphosphotyrosine matrix. Also, elution of EGF-increased phospholipase C activity was accomplished by either phosphotyrosine or the antibody-recognized hapten phenylphosphate, but not by phosphothreonine, phosphoserine, or tyrosine (*10*). We conclude that this immunoaffinity matrix adsorbs phospholipase C activity through specific interactions with phosphotyrosine-containing proteins.

Table 1. Subcellular localization of phosphotyrosine-immunopurified phospholipase C activity. A-431 cells were treated without or with EGF (200 ng/ml for 5 minutes) and prepared as described (*13*), except that 0.5 ml per plate of ice-cold homogenization buffer (solubilization buffer without octylglucoside) was added, and the cells were collected by scraping and lysed by 40 brisk strokes with a Teflon Wheaton homogenizer. The nuclear pellet was removed by centrifugation at 300*g* for 5 minutes. The post-nuclear supernatant was centrifuged at 240,000*g* for 30 minutes to separate the cytosolic and membrane fractions. Both cytosolic and membrane fractions were solubilized in 1% octylglucoside, and the equivalent of one plate of cells (cytosol, 450 μg of protein; membrane, 175 μg of protein) was adsorbed to the antiphosphotyrosine matrix. Phenylphosphate eluates were obtained, and samples (20 μl) were assayed as described (Fig. 1A) for phospholipase C activity. The results are presented as the amount of phospholipase C activity in the total eluate (200 μl) and are measured as picomoles of IP_3 formed in 15 minutes. Radioactivity present in the acid supernatant of buffer-only control reaction mixtures (175 cpm) was subtracted, and the calculation of IP_3 formation was based on substrate specific activity (2480 cpm/nmol).

Preparation	Eluate phospholipase C activity (pmol/15 min)	
	Without EGF	With EGF
Membrane	24	16
Cytosol	44	823

Our previous study (*3*) demonstrated that intracellular Ca^{2+} was necessary for EGF-stimulated IP_3 formation in intact A-431 cells. The Ca^{2+} requirement of phospholipase C activity present in the antiphosphotyrosine eluates from control and EGF-treated cells was assessed by performing in vitro assays in the presence of increasing concentrations of free Ca^{2+}. Maximal stimulation of phospholipase C activity (15-fold) was obtained with approximately 1 μ*M* Ca^{2+}, and half-maximal activation was achieved with 200 n*M* Ca^{2+}. In the absence of Ca^{2+} little phospholipase C activity was evident and no difference between control and EGF-treated preparations was noted (*10*).

To characterize further the immunoisolated phospholipase C activity, we analyzed substrate selectivity. At the concentration of substrates assayed (200 μ*M*), PIP_2 was the predominant substrate hydrolyzed by eluates from EGF-treated cells (63 pmol/15 min per 20 μl of eluate). A small amount of PIP was hydrolyzed (5 pmol/15 min per 20 μl of eluate), but essentially no hydrolysis of phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine was detected (<1 pmol/15 min per 20 μl of eluate). Therefore, the immunoisolated phospholipase C activity from EGF-stimulated cells is relatively selective for PIP_2.

To determine the subcellular origin of the phospholipase C activity recovered in the antiphosphotyrosine eluates, we homogenized control and EGF-treated cells and fractionated them into membrane and cytosolic preparations. These fractions were then solubilized with octylglucoside, adsorbed to the antibody matrix, and washed. The phenylphosphate eluates were assayed for activity (Table 1). The data show that EGF-sensitive phospholipase C activity isolated with the antiphosphotyrosine is found in the cytosolic fraction. No EGF-sensitive phospholipase C activity was detected in the membrane fraction. The most straightforward interpretation of these data is that EGF treatment of A-431 cells increased the level of phospholipase C activity recovered from the cytosolic fraction.

Our data indicate that a significantly increased level of a PIP_2-selective, Ca^{2+}-dependent phospholipase C activity can be recovered from EGF-treated A-431 cells with an antibody to phosphotyrosine. Since our previous results (*3*) show that cells with high levels of EGF receptors produce inositol phosphates in response to EGF, we tested several other carcinoma cell lines (NA, HSC-1, HSC-2, CA-922, and MDA-468) that overexpress the EGF receptor for EGF-stimulated phospholipase C activity in vitro and found similar results (*10*).

Proteins could be specifically adsorbed to the immunoaffinity matrix through association with other phosphotyrosine-containing proteins, such as the EGF receptor—the major phosphotyrosine-containing protein in A-431 cells. Several findings show that this is unlikely. First, the EGF-sensitive phospholipase C activity obtained with the antiphosphotyrosine matrix is recovered from cytosol (Table 1), whereas the EGF receptor (an integral membrane protein) is present in the membrane fraction (*5*). In addition, when crude extracts from EGF-stimulated A-431 cells were first adsorbed to either wheat germ lectin– or EGF-agarose, both of which bind the EGF receptor (*11*), the EGF-sensitive phospholipase C activity was subsequently recovered from the nonadsorbed supernatant, showing that the activi-

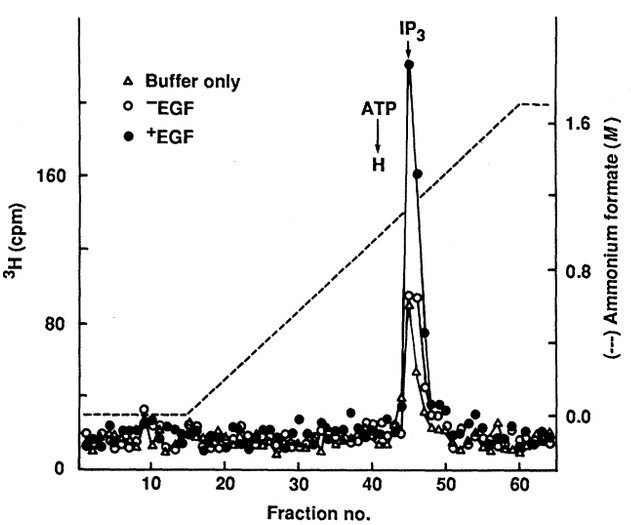

Fig. 2. Separation by high-performance liquid chromatography (HPLC) of water-soluble products formed in the in vitro phospholipase C assay. The phospholipase C assay was performed as described in Fig. 1 with 20 μl of elution buffer only (△) or 20 μl of the antiphosphotyrosine eluate from untreated (○) or EGF-treated (●) cells. Ether-extracted supernatants were loaded onto an HPLC column (Partisil 10 SAX), and the reaction products were eluted with a 15-minute 0 to 1.7*M* ammonium formate (*p*H 3.7) linear gradient. The flow rate was 1.6 ml/min, and 0.5-ml fractions were collected and assayed for radioactivity. ATP was added as an internal standard to each sample and its elution position, monitored at 254 nm, is indicated. The data represent the average of duplicate assays that varied by less than 5%.

ty was not associated with the EGF receptor (*10*).

Neither adenosine triphosphate (ATP) nor bradykinin, both of which stimulate formation of IP_3 in A-431 cells, enhanced the recovery of phospholipase C activity from the phosphotyrosine antibody matrix (*10*). This finding suggests that these agonists induce the formation of IP_3 by a mechanism or mechanisms that do not involve tyrosine phosphorylation. While our experiments have not focused on possible roles for G proteins in mediating EGF-stimulated formation of IP_3, we have been unable to further activate the immunoaffinity-purified phospholipase C by addition of guanosine triphosphate (GTP) or GTP-γ-S. Also, treatment of A-431 cells with toxins or other agents that modify certain G proteins does not affect the EGF-stimulated formation of inositol phosphates in vivo (*10*).

To date, although several proteins, including several enzymes, have been identified in vivo or in vitro as possible exogenous substrates of tyrosine kinases (*5*), none of these is a good candidate as a molecule regulating cell proliferation. In no case has a biochemical function been associated with tyrosine phosphorylation of exogenous substrates. Data obtained in vivo by expression of tyrosine kinase–defective mutants of the EGF receptor indicate that all events distal to receptor occupancy, including the formation of inositol phosphates and the release of stored intracellular Ca^{2+} (*12*), are abrogated directly or indirectly as a consequence of this mutation. Those data support the hypothesis that tyrosine kinase activity is required for EGF-stimulated PIP_2 turnover in vivo, but do not discriminate as to whether autophosphorylation of the receptor or exogenous substrate phosphorylation is critical to the generation of biological responses. Our findings suggest that either phospholipase C or a tightly associated protein is an exogenous substrate for EGF-stimulated tyrosine phosphorylation, and this tyrosine phosphorylation may have functional consequences relating to PIP_2 hydrolysis.

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13. A-431 cells were subcultured in 100-mm Falcon tissue culture plates at a density of 1×10^4 to 2.5×10^4 cell/cm^2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum, 20 m*M* Hepes (*p*H 7.4), and gentamicin (50 mg/ml) and grown for 2 to 3 days to a density of 5×10^4 to 10×10^4 cell/cm^2. Then the cells were washed twice with DMEM and incubated at 37°C in 4 ml of DMEM. Sodium orthovanadate was added to all cultures to a final concentration of 100 μ*M* for 10 minutes, and the cultures were treated without or with EGF (200 ng/ml) for an additional 0 to 60 minutes. (A control experiment demonstrated that treatment with vanadate prior to growth factor addition was not required.) Subsequently, the cultures were placed on ice and washed four times with ice-cold calcium- and magnesium-free phosphate-buffered saline. Soluble cell extracts were prepared by adding 1.4 ml per plate of ice-cold solubilization buffer [20 m*M* Hepes, *p*H 7.2, 1.0% octyl β-D-glucopyranoside, 30 m*M* sodium pyrophosphate, 50 m*M* sodium chloride, 5 m*M* β-glycerophosphate, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonylfluoride, 100 μ*M* sodium orthovanadate, aprotinin (10 μg/ml), and leupeptin (10 μg/ml)]. The soluble cell extracts were centrifuged for 10 minutes at 250,000*g*. The clarified extracts (0.75 to 1.5 mg/ml) were treated with 50 to 100 μl of Sepharose-linked phosphotyrosine antibody (1G2) at a ratio of approximately 1.0 to 1.5 mg extract to 100 μl of packed bead matrix. After adsorption to the matrix for 2 hours at 4°C, the bead matrix was batch-washed four times with ten volumes of ice-cold solubilization buffer. Finally, eluates from the matrix were obtained by addition of 1.5 volumes of elution buffer (solubilization buffer plus 1 m*M* phenylphosphate). After 10 minutes at 4°C the eluates (100 to 200 μl) were recovered by centrifugation into a second tube. The phospholipase C assay was performed by adding 20 μl of the eluates to 30 μl of a reaction solution containing [^{3}H]PIP_2, octylglucoside, calcium chloride plus EGTA, sodium phosphate (*p*H 6.8), and potassium chloride, yielding, in a 50-μl volume, final concentrations of 200 μ*M*, 0.65%, 1 μ*M* free Ca^{2+}, 20 m*M*, and 40 m*M*, respectively.

14. We thank S. Heaver and D. Sullins for their assistance in typing this manuscript and K. Adair for technical assistance. This research was supported by NIH medical scientist training grant GM 07347 (to M.I.W.), by NCI grant CA 43720, and by NIH grant DK 38517.

28 April 1988; accepted 5 July 1988

Autoregulation of Enzymes by Pseudosubstrate Prototopes: Myosin Light Chain Kinase

RICHARD B. PEARSON, RICHARD E. H. WETTENHALL, ANTHONY R. MEANS, DAVID J. HARTSHORNE, BRUCE E. KEMP*

The myosin light chain kinase requires calmodulin for activation. Tryptic cleavage of the enzyme generates an inactive 64-kilodalton (kD) fragment that can be further cleaved to form a constitutively active, calmodulin-independent, 61-kD fragment. Microsequencing and amino acid analysis of purified peptides after proteolysis of the 61- and 64-kD fragments were used to determine the amino-terminal and carboxyl-terminal sequences of the 64-kD fragment. Cleavage within the calmodulin-binding region at Arg^{505} generates the catalytically inactive 64-kD fragment, which is incapable of binding calmodulin. Further digestion removes a carboxyl-terminal fragment, including the pseudosubstrate sequence Ser^{484}-Lys-Asp-Arg-Met-Lys-Lys-Tyr-Met-Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg^{505} and results in a calmodulin-independent 61-kD fragment. Both the 61- and 64-kD fragments have the same primary amino-terminal sequences. These results provide direct support for the concept that the pseudosubstrate structure binds the active site and that the role of calmodulin is to modulate this interaction. Pseudosubstrates may be utilized in analogous ways by other allosterically regulated enzymes.

PROTEIN KINASES ARE INVOLVED IN the regulation of protein and enzyme functions necessary for numerous biological processes (*1*). The smooth muscle myosin light chain kinase (MLCK) plays an obligatory regulatory role in the initiation of smooth muscle contraction (*2*). In many instances, protein kinases are inactive and require activation by regulators. In the case of the MLCK, the regulator is calmodulin. Structural studies by Blumenthal *et al.* (*3*) showed that a peptide segment of 26 residues was responsible for binding calmodulin

R. B. Pearson and B. E. Kemp, Department of Medicine, University of Melbourne, Repatriation General Hospital, Heidelberg, Victoria 3081, Australia.
R. E. H. Wettenhall, Department of Biochemistry, La Trobe University, Bundoora, Victoria, Australia.
A. R. Means, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.
D. J. Hartshorne, Department of Nutrition and Food Science, University of Arizona, Tucson, AZ 85721.

*To whom correspondence should be addressed.