ter City, CA). Primary antisera used were monoclonal antibody to rat neurofilament (Labsystems, Helsinki, Finland) at 1:75 dilution (Fig. 3A), monoclonal antibody to rat neurofilament antibody SMI 33 (Sternberger-Meyer, Jarrettsville, MD) at 1:2000 dilution (Fig. 3B); rabbit antibody to NSE (Incstar, Stillwater, MN) at a dilution of 1:2 (Fig. 3C); and rabbit antibody to GFAP (DAKO-Patts, Santa Barbara, CA) at 1:800 dilution (Fig. 3D). There was negligible nonspecific staining where immune primary serum was deleted or replaced by nonimmune serum. In addition, specificity of staining for each antibody was checked with rat brain control slides and by protein immunoblotting (*5*). Cells are fixed for immunohistochemistry when they are about 50% differentiated because, if all cells are allowed to differentiate, they tend to lift off the slides during fixation.

12. S-M. Hsu, L. Raine, M. Fargen, *J. Histochem. Cytochem.* **29**, 577 (1981).

13. Supported by USPHS grants MH 18501 and DA 00266, Research Scientist Award DA 00074 to S.H.S., a gift from the Bristol-Myers–Squibb Company, and USPHS grant NS 01231 to G.V.R. We thank M. L. Oster-Granite for critical review of the data.

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Identity of Inositol 1,2-Cyclic Phosphate 2-Phosphohydrolase with Lipocortin III

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The amino acid sequences of three fragments of cyanogen bromide–digested human placental inositol 1,2-cyclic phosphate 2-phosphohydrolase, an enzyme of the phosphatidylinositol signaling pathway, are identical to sequences within lipocortin III, a member of a family of homologous calcium- and phospholipid-binding proteins that do not have defined physiological functions. Lipocortin III has also been previously identified as placental anticoagulant protein III (PAP III) and calcimedin 35$_\alpha$. Antibodies to PAP III detected PAP III and inositol 1,2-cyclic phosphate 2-phosphohydrolase with identical reactivity on immunoblotting. In addition, inositol 1,2-cyclic phosphate 2-phosphohydrolase was stimulated by the same acidic phospholipids that bind lipocortins.

THE TURNOVER OF INOSITOL PHOSpholipids produces messenger molecules, including inositol phosphates, diacylglycerol, and eicosanoids, in response to a variety of extracellular effectors (*1*). Phosphatidylinositol (PI)-specific phospholipase C hydrolyzes PI, phosphatidylinositol 4-phosphate (PIP), and phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce both cyclic and noncyclic inositol phosphates (*2*). Inositol 1,2-cyclic phosphate is derived directly from phospholipase C cleavage of PI or indirectly via phospholipase C cleavage of PIP and PIP_2, with subsequent enzymatic removal of the phosphates from positions 4 and 5 of the inositol ring (*2, 3*). The existence of inositol cyclic phosphate in vivo has been shown in a variety of tissues (*4*), and carbachol stimulation of pancreatic minilobules leads to a 20- to 400-fold increase in inositol 1,2-cyclic phosphate (*5*), as well as an increase in cyclic inositol 1,2,4-bisphosphate and cyclic inositol 1,2,4,5-trisphosphate (*4, 5*). Inositol cyclic phosphates accumulate in stimulated cells for longer periods than the inositol phosphates (*4*), suggesting their importance in evoking sustained or delayed responses, including stimulation of cell growth. Cyclic inositol 1,2-phosphate 2-phosphohydrolase cleaves the cyclic bond of inositol 1,2-cyclic phosphate to form inositol 1-phosphate. Because all inositol cyclic phosphates are metabolized through this reaction, it is likely that this enzyme regulates the cellular concentrations of these molecules. This enzyme is widely distributed (*6*) and has been isolated and characterized from human placenta (*7*).

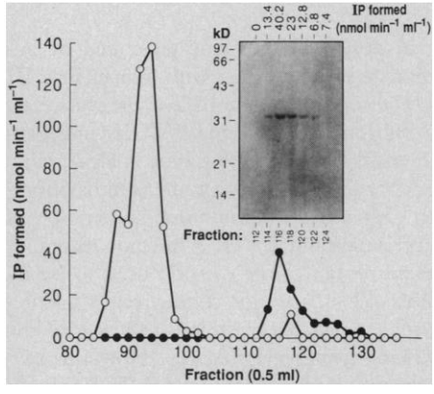
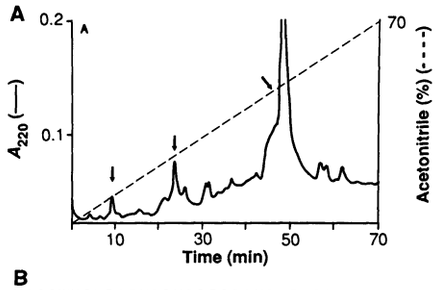


Fig. 1. Inositol 1,2-cyclic phosphate phosphohydrolase activity after gel filtration in the presence (closed circles) or absence (open circles) of 100 m*M* NaCl. (**Inset**) An SDS–12% polyacrylamide gel showing the correlation of a 33-kD band with enzyme activity from the final gel filtration step in the presence of NaCl. The column is a Biosil-TSK 250 size-exclusion (three columns in series, 7.5 mm by 600 mm each) column and was equilibrated with 20 m*M* 2-[*N*-morpholino]ethanesulfonic acid (MES) (*p*H 6.5) in the absence or presence of 100 m*M* NaCl. Activity was eluted with a flow rate of 0.5 ml/min of the same buffer. All assays were done in the presence of alkaline phosphatase as described (7). IP, inositol 1-phosphate.



B

MASIWVGHRGTVRDYPDFSPSVDAEAIQKAIRG
IGTDEKMLISILTERSNAQRQLIVKEYQAAYGK
ELKDDLKGDLSGHFEHLMVALVTPPAVFDAKQL
KKSMKGAGTNEDALIEILTTRTSRQMKDISQAY
YTVYKKSLGDDISSETSGDFRKALLTLADGRRD
ESLKVDEHLAKQDAQILYKAGENRWGTDEDKFT
EILCLRSFPQLKLTFDEYRNISQKDIVDSIKGE
LSGHFEDLLLAIVNCVRNTPAFLAERLHRALKG
IGTDEFTLNRIMVSRSEIDLLDIRTEFKKHYGY
SLYSAIKSDTSGCYEITLLKICGGDD

Fig. 2. Partial amino acid sequence of inositol 1,2-cyclic phosphate 2-phosphohydrolase is identical to lipocortin III amino acid sequence. (**A**) Purified phosphohydrolase (~12 μg, 340 pmol) was lyophilized and treated with CNBr (~1 mg) in 70% trifluoroacetic acid (TFA) for 24 hours at 23°C in the dark under an N_2 atmosphere. The sample was then dried under N_2, degassed, dissolved in 50% TFA, and injected onto a reversed-phase 300 column (2.1 mm by 30 mm) on an Applied Biosystems microbore HPLC. A linear gradient of 0 to 90% acetonitrile in 0.1% TFA at a flow rate of 0.1 ml/min over 90 min was used to elute the peptides. Peaks were detected by absorbance at a wavelength of 220 nm (A_{220}). The arrows indicate peaks that yielded amino acid sequence. (**B**) The predicted amino acid sequence of lipocortin III and the sequence obtained on an Applied Biosystems gas-phase sequencer from the CNBr fragments of inositol cyclic 1,2-phosphate 2-phosphohydrolase. The sequenced peptides are underlined and numbered. Peptide 3 was sequenced twice from two different preparations. The yield of amino acids ranged from 60 to 100 pmols. The single-letter code is used for amino acids (*20*).

For the current study, we modified our previous procedure for the isolation of the phosphohydrolase (*7*) and obtained material that gave a single band after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) as before, but with higher specific activity (5 to 20 versus 2 μmol min^{-1} mg^{-1}). We attribute this difference to preservation of activity by the more speedy purification (*8*). When the enzyme is subjected to chromatography by gel filtration in the absence of NaCl, the peak of activity is heterogeneous and elutes at a position corresponding to a molecular size of ~55 kD; however, after the addition of 100 m*M* NaCl to the buffer, the enzyme elutes at a position correspond-

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Table 1. The predicted amino acid composition of lipocortin III is similar to the amino acid compositions of PAP III and inositol 1,2-cyclic phosphate 2-phosphohydrolase. The lipocortin III sequence was predicted from the published cDNA sequence (*10*). For PAP III, actual amino acid analysis results were obtained from the preparation of PAP III used in the immunoblot analysis of Fig. 3. For the phosphohydrolase, actual amino acid analysis results were obtained from the preparation shown in the inset of Fig. 1 and in the slot-blot analysis of Fig. 3. Samples (2.0 μg) were hydrolyzed with 6*N* HCl in sealed evacuated glass tubes at 110°C. Analysis was performed on a Waters programmable-gradient HPLC with post-column *o*-phthalaldehyde detection with continuous hypochlorite infusion for the determination of proline. ND, not determined.

Amino acid	Residues per molecule of		
	Lipocortin III	PAP III	Inositol 1,2-cyclic phosphate 2-phosphohydrolase
A	23	24	24
R	19	21	19
N + D	37	38	37
C	3	ND	ND
Q + E	33	34	35
G	22	26	23
H	7	7	7
I	24	22	20
L	35	35	34
K	26	26	25
M	6	3	2
F	11	11	12
P	6	6	6
S	24	24	21
T	20	19	18
W	2	ND	ND
Y	12	12	11
V	13	12	14

ing to 20 to 25 kD (Fig. 1). The two gel-filtration steps (in the absence and then the presence of salt) are critical for obtaining a homogeneous preparation of active enzyme. The size of the enzyme, as determined by SDS-PAGE (Fig. 1, inset), is approximately 33 kD rather than the 29 kD reported previously (*7*). We have carried out this modified purification four times with similar results.

Inositol 1,2-cyclic phosphate 2-phosphohydrolase (12 μg) was cleaved with cyanogen bromide (CNBr) (*9*), and the fragments were separated by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 2A) and subjected to sequence analysis. The amino acid sequences obtained were identical to sequences within lipocortin III (*10*) (Fig. 2B). In addition, the amino acid composition of the phosphohydrolase is nearly identical to that predicted from the cDNA of human lipocortin III and to that obtained from analysis of placental anticoagulant protein III (PAP III) (Table 1).

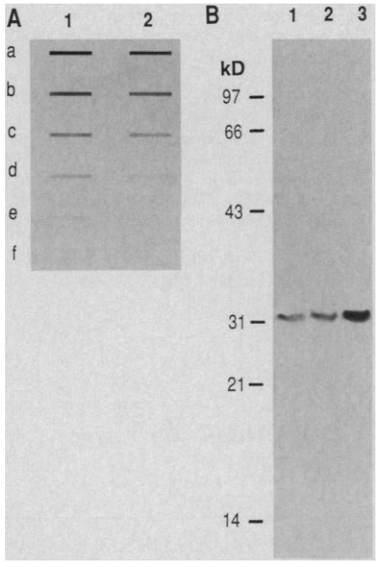

Fig. 3. PAP III and inositol 1,2-cyclic phosphate 2-phosphohydrolase immunoblot by use of an antibody generated to PAP III. (**A**) Equal amounts of PAP III (*12*) or the phosphohydrolase (*7*) and a constant amount of bovine serum albumin (BSA) (0.5 μg) were loaded onto nitrocellulose on a Bio-Rad slot-blot apparatus. Alkaline phosphatase–conjugated goat antibodies to rabbit immunoglobulin G (Promega) were used for the detection system. The amount of phosphohydrolase or PAP III in each row is as follows: a, 50 ng; b, 25 ng; c, 12.5 ng; d, 6 ng; e, 3 ng; and f, 1.5 ng. Column 1, phosphohydrolase; column 2, PAP III. (**B**) PAP III (lane 1, 50 ng) and two different preparations of the phosphohydrolase (lanes 2 and 3, 50 and 200 ng, respectively) were separated by electrophoresis through a SDS–12% polyacrylamide gel, transferred to nitrocellulose, and developed as described in (A).

A polyclonal antibody generated by immunization of a rabbit with human PAP III (*11*) recognizes inositol 1,2-cyclic phosphate 2-phosphohydrolase and PAP III with equal intensity by either slot blot analysis (Fig. 3A) or immunoblotting after electrophoresis (Fig. 3B); a nonimmune preparation of immunoglobin G (IgG) did not recognize these proteins. The method used to purify PAP III (*12*) is not conducive to maintenance of phosphohydrolase activity (the low *p*H inactivates the enzyme). However, two separate preparations of PAP III did retain activity (2.0 and 0.03 μmol min^{-1} mg^{-1}). Assay of a preparation of rat calcimedin 35_{α} (*13*) also retained activity (0.02 μmol min^{-1} mg^{-1}).

Inositol 1,2-cyclic phosphate 2-phosphohydrolase was stimulated by phosphatidylserine, phosphatidic acid, and phosphatidylinositol, but not by phosphatidylcholine, phosphatidylethanolamine, or diacylglycerol (Fig. 4) when assayed in the presence of 0.375 m*M* $MnCl_2$. This profile of lipid activation is similar to that described for the

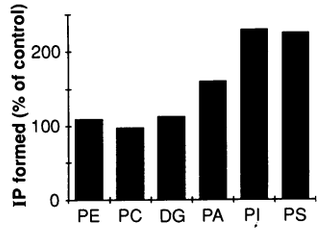

Fig. 4. Activation of inositol 1,2-cyclic phosphate 2-phosphohydrolase by lipids. All lipids were dissolved in tris-buffered saline and sonicated at 100 W for 30 s at 4°C. Control has no lipid added to the assay. Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and phosphatidylserine (PS) were at a concentration of 25 μg/ml. Diacylglycerol (DG) was at 0.5 μg/ml and phosphatidic acid (PA) was 75 μg/ml. All assays, except for the condition containing PA (assayed as described in Fig. 5), were performed in the absence of alkaline phosphatase under the conditions containing 0.375 m*M* $MnCl_2$ as described (*7*). Each condition was reproduced 6 to 20 times with a SD ranging from 5 to 10%, except for PI which was 19%.

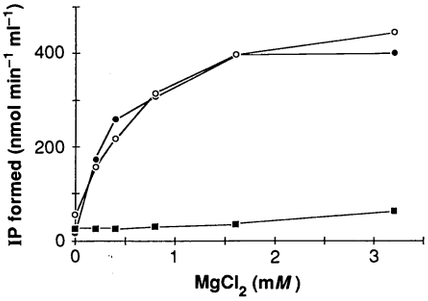

Fig. 5. Activation of inositol 1,2-cyclic phosphate 2-phosphohydrolase by PA and PS is dependent on $MgCl_2$. Open circles, enzyme activity in the presence of PS (25 μg/ml); closed circles, enzyme activity in the presence of PA (75 μg/ml); and closed squares, enzyme activity in the absence of added lipid. Lipids were prepared as described in Fig. 4. All assays were done in the presence of alkaline phosphatase with a phosphohydrolase concentration of 10 μg/ml as described (*7*). The data presented are the average of two experiments in duplicate (SD ranged from 5 to 10%).

lipid-binding activity of lipocortins (*14*). When the enzyme activity is measured with $MgCl_2$ in place of $MnCl_2$, the activation by phosphatidylserine and phosphatidic acid is much greater (Fig. 5). Together these physicochemical, immunological, and functional data show that lipocortin III is identical to inositol 1,2-cyclic phosphate 2-phosphohydrolase.

The family of proteins variously designated as annexins, lipocortins, calpactins, calcimedins, and placental anticoagulant proteins has at least eight members that share 40 to 60% amino acid identity in a core domain that is repeated four to eight times in each protein (*15*). These proteins also share the properties of binding Ca^{2+} and phospholip-

ids. Although multiple functions have been suggested for these proteins, their true physiological roles have not yet been elucidated. They have been implicated in phospholipase A_2 regulation (*16*), membrane trafficking (*17*), cytoskeletal organization (*18*), and blood coagulation (*19*). In many cases the proposed function requires that the proteins be secreted, yet these proteins lack signal sequences and are found within cells. In addition, many of the ascribed functions can be attributed to the metal- or lipid-binding properties of these molecules, and large amounts of protein are needed. In contrast, inositol 1,2-cyclic phosphate 2-phosphohydrolase activity is catalytic with 700 mol of product formed per minute per mole of protein, suggesting that this is a physiological function. The fact that phosphohydrolase activity is affected by divalent metals has been noted previously (*7*). We now show that acidic phospholipids stimulate the enzyme.

There are numerous enzymes (more than 15) that have been shown to metabolize the water-soluble inositol phosphates produced during PI turnover. It is possible that other lipocortins are also enzymes that change the concentrations of messenger molecules during cellular activation.

REFERENCES AND NOTES

1. R. H. Michell, C. J. Kirk, L. M. Jones, C. P. Downes, J. A. Creba, *Philos. Trans. R. Soc. London Ser.B* **296**, 123 (1981); L. E. Hokin, *Annu. Rev. Biochem.* **54**, 205 (1985); P. W. Majerus *et al.*, *Science* **234**, 1519 (1986); P. W. Majerus *et al.*, *J. Biol. Chem.* **263**, 3051 (1988); M. J. Berridge and R. F. Irvine, *Nature* **341**, 197 (1989).
2. D. B. Wilson, T. E. Bross, W. R. Sherman, R. A. Berger, P. W. Majerus, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4013 (1985); J. W. Kim, S. H. Ryu, S. G. Rhee, *Biochem. Biophys. Res. Commun.* **163**, 177 (1989).
3. T. M. Connolly, D. B. Wilson, T. E. Bross, P. W. Majerus, *J. Biol. Chem.* **261**, 122 (1986).
4. M. A. Koch and H. Diringer, *Biochem. Biophys. Res. Commun.* **58**, 361 (1974); J. F. Dixon and L. E. Hokin, *J. Biol. Chem.* **260**, 16068 (1985); J. A. Shayman, R. J. Auchus, A. R. Morrison, *Arch. Biochem. Biophys.* **886**, 171 (1986); S. H. Binder, P. C. Weber, W. Siess, *Anal. Biochem.* **148**, 220 (1985); H. Ishii, T. M. Connolly, T. E. Bross, P. W. Majerus, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6397 (1986); R. A. Graham, R. A. Meyer, B. S. Szwergold, T. R. Brown, *J. Biol. Chem.* **262**, 35 (1987); M. C. Sekar, J. F. Dixon, L. E. Hokin, *ibid.*, p. 340; S. J. F. Dixon and L. E. Hokin, *Biochem. Biophys. Res. Commun.* **149**, 1208 (1987); A. R. Hughes, H. Takemura, J. W. Putney, Jr., *J. Biol. Chem.* **263**, 10314 (1988).
5. J. F. Dixon and L. E. Hokin, *J. Biol. Chem.* **262**, 13892 (1987).
6. M. C. Dawson and N. G. Clarke, *Biochem. J.* **127**, 113 (1972); M. C. Dawson and N. G. Clarke, *ibid.* **134**, 59 (1973).
7. T. S. Ross and P. W. Majerus, *J. Biol. Chem.* **261**, 11119 (1986).
8. This purification described in (*7*) has been modified slightly. The first step, which consisted of multiple HPLC runs on DEAE columns has now been replaced with one run on a preparative BioGel TSK DEAE-5PW column (55 mm by 200 mm) from Bio-Rad and the third step (gel filtration 2) has been omitted.
9. E. Gross, *Methods Enzymol.* **11**, 238 (1967).
10. R. B. Pepinsky *et al.*, *J. Biol. Chem.* **263**, 10799 (1988).
11. PAP III (100 μg) was injected into a rabbit in complete Freund's adjuvant and then injected twice at 2-week intervals in incomplete Freund's adjuvant. Immunoglobulin G was prepared by ammonium sulfate precipitation followed by DEAE chromatography at *p*H 5.0 [N. Harboe and A. Ingild, *Scand J. Immunol.* **2**, 161 (1973)].
12. J. F. Tait *et al.*, *Biochemistry* **27**, 6268 (1988).
13. M. A. Kaetzel, P. Hazarika, J. R. Dedman, *J. Biol. Chem.* **264**, 14463 (1989).
14. J. Glenney, *ibid.* **261**, 7247 (1986); J. F. Tait, D. Gibson, K. Fujikawa, *ibid.* **264**, 7944 (1989); J. F. Tait and D. Gibson, *Cytokines and Lipocortins in Inflammation and Differentiation*, L. Parente, Ed. (Liss, New York, in press).
15. M. R. Crompton, S. E. Moss, M. J. Crumpton, *Cell* **55**, 1 (1988); H. T. Haigler, J. M. Fitch, D. D. Schlaepfer, *Trends Biochem. Sci.* **14**, 48 (1989).
16. R. J. Flower and G. J. Blackwell, *Nature* **278**, 456 (1979); G. J. Blackwell *et al.*, *ibid.* **287**, 147 (1980).
17. C. E. Creutz, *Biochem. Biophys. Res. Commun.* **103**, 1395 (1981); M. J. Geisow and R. D. Burgoyne, *J. Neurochem.* **38**, 1735 (1982); T. C. Sudhof, J. H. Walker, J. Obrocki, *EMBO J.* **1**, 1167 (1982).
18. V. Gerke and K. Weber, *EMBO J.* **3**, 227 (1984); J. R. Glenney, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7884 (1986).
19. C. P. M. Reutelingsperger, G. Hornstra, H. C. Hemker, *Eur. J. Biochem.* **151**, 625 (1985); T. Funakoshi, R. L. Heimark, L. E. Hendrickson, B. A. McMullen, K. Fujikawa, *Biochemistry* **26**, 5572 (1987); S. Kondo, M. Noguchi, T. Funakoshi, K. Fujikawa, W. Kisiel, *Thromb. Res.* **48**, 449 (1987); A. Iwasaki *et al.*, *J. Biochem.* **102**, 1261 (1987).
20. Abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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Effect of Phospholipase C–γ Overexpression on PDGF-Induced Second Messengers and Mitogenesis

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Platelet-derived growth factor (PDGF) stimulates phospholipase C (PLC) activity and the phosphorylation of the γ isozyme of PLC (PLC-γ) in vitro and in living cells. The role of PLC-γ in the phosphoinositide signaling pathway was addressed by examining the effect of overexpression of PLC-γ on cellular responses to PDGF. Overexpression of PLC-γ correlated with PDGF-induced tyrosine phosphorylation of PLC-γ and with PDGF-induced breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2). However, neither bradykinin- nor lysophosphatidic acid–induced phosphoinositide metabolism was enhanced in the transfected cells, suggesting that the G protein–coupled phosphoinositide responses to these ligands are mediated by other PLC isozymes. The enhanced PDGF-induced generation of inositol trisphosphate (IP_3) did not enhance intracellular calcium signaling or influence PDGF-induced DNA synthesis. Thus, enzymes other than PLC-γ may limit PDGF-induced calcium signaling and DNA synthesis. Alternatively, PDGF-induced calcium signaling and DNA synthesis may use biochemical pathways other than phosphoinositide metabolism for signal transduction.

THE BREAKDOWN OF PIP_2 BY PLC generates at least two intracellular messengers: inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$], which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C (*1, 2*). Growth factors such as PDGF and epidermal growth factor (EGF) activate PLC, and this activation requires the intrinsic tyrosine kinase activity of the growth factor receptor (*3*). These growth factors stimulate the phosphorylation of one of the PLC isozymes, PLC-γ (*4–6*). This most likely results from a direct interaction of the growth factor receptors with PLC-γ, as purified EGF or PDGF receptors can directly phosphorylate PLC-γ on tyrosine residues (*6, 7*) and PLC-γ is associated with these receptors in growth factor–stimulated cells (*6, 8*). However, it is not clear whether PLC-γ is actually involved in the growth factor stimulation of PLC activity or whether phosphoinositide metabolism has a primary signaling role in mitogenesis.

To examine this issue, we overexpressed PLC-γ in cells by cotransfecting a mammalian expression vector containing bovine PLC-γ cDNA together with pSVneo into NIH 3T3 cells and selected clones by their resistance to geneticin (G418) (*9*). Immunoblotting with antibodies to PLC-γ (anti–PLC-γ) after PDGF treatment showed in-

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