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Enhanced phospholipase C-γ1 activity produced by association of independently expressed X and Y domain polypeptides

(SH region/protein:protein interaction)

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ABSTRACT **The X and Y domains of phospholipase C (PLC)-γ1, which are conserved in all mammalian phosphoinositide-specific PLC isoforms and are proposed to interact to form the catalytic site, have been expressed as individual hexahistidine-tagged fusion proteins in the baculovirus system. Following coinfection of insect cells with recombinant viruses, association of X and Y polypeptides was demonstrated in coprecipitation assays. When enzyme activity was examined, neither domain possessed catalytic activity when expressed alone; however, coexpression of the X and Y polypeptides produced a funtional enzyme. This reconstituted phospholipase activity remained completely dependent on the presence of free Ca^{2+}. The specific activity of the X:Y complex was significantly greater (20- to 100-fold) than that of holo-PLC-γ1 and was only moderately influenced by varying the concentration of substrate. The enzyme activities of holo-PLC-γ1 and the X:Y complex exhibited distinct pH optima. For holoPLC-γ1 maximal activity was detected at pH 5.0, while activity of the X:Y complex was maximal at pH 7.2.**

Mammalian phosphoinositide-specific phospholipase C enzymes (PLCβ, PLCγ, and PLCδ) hydrolyze the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), generating two second messenger molecules, inositol 1,4,5-trisphosphate and diacylglycerol (1). Members of the PLC family include four PLC-β isoforms, which are regulated by heterotrimeric G proteins, and two PLC-γ isoforms, which are activated by tyrosine kinases. Additionally, there are four members of the PLC-δ family; however, regulation of the activity of these isoforms is unclear. All isoforms share only two regions of conserved sequence, termed the X domain (≈145 residues) and the Y domain (≈245 residues), which, based on deletion analyses, are thought to be required for PLC catalytic activity (2–5). Site-directed mutagenesis studies have shown that several conserved residues in the X domain are essential for catalytic activity (6). Proteolysis studies of immunopurified PLC-γ1 (7) or purified brain PLC-δ (4, 5) have demonstrated that separate polypeptides containing X and Y domains remain physically associated but the molecular nature of and the requirements for the association are unknown (4, 5).

PLC-γ isozymes uniquely contain two src homology (SH)2 domains and one SH3 domain intervening in the primary sequence between the X and Y domains (8). The SH2 domains are known to mediate association of PLC-γ isoforms with tyrosine phosphorylated proteins, such as growth factor receptor tyrosine kinases (9, 10), and the SH3 domain facilitates interactions with proteins that have proline-rich sequences, such as dynamin (11, 12). The physiological significance of the latter is not clear, while the former is essential for tyrosine phosphorylation of PLC-γ1 (13).

In addition to mediating protein:protein interactions, the SH region may play a role in the intramolecular regulation of PLC-γ1 activity. For example, increased PLC-γ1 activity has been reported to occur following tyrosine phosphorylation (14) or proteolysis (7) within the SH region, or upon binding of tyrosine phosphorylated peptides to SH2 domains (15). Also, overexpression of SH region sequences in intact cells has been reported (16) to increase PLC-γ activity. In contrast, inhibition of basal PLC-γ activity has been demonstrated in SH region deletion mutants (2, 3) and by the addition of an SH peptide to PLC-γ holoenzyme (17, 18). Hence, there is evidence for an influence, though not well defined, of the SH region on basal PLC-γ catalytic activity. We have explored the intrinsic capacity of the X and Y domains of PLC-γ1 to associate and have assessed the relative catalytic activity of these conserved domains, individually or together, in the absence of the SH region.

MATERIALS AND METHODS

Construction of Recombinant Baculoviruses. The cDNA encoding rat PLC-γ1 (generously provided by Sue Goo Rhee, National Institutes of Health) was cut with *Bam*HI and *Bgl*II restriction endonucleases to generate a 1550-nucleotide (nt) fragment encoding the N terminus of PLC-γ1 through the X domain (residues 1–516). This fragment was subcloned into the transfer vector pBlueBacHis (Invitrogen) so that the coding sequence was in frame with the sequences encoding a hexahistidine tag. To generate a fragment encoding the Y domain through the C-terminus, a 2300-nt *Sph*I/*Bam*HI fragment was subcloned into m13 mp19 RF DNA. A 1500-nt fragment was excised with *Hin*dIII (encoding residues 902-1290) was subcloned into pBlueBacHis and the orientation and reading frames of the transfer vectors were determined by dideoxy sequencing (United States Biochemical). These transfer plasmids and linearized Baculogold viral DNA (PharMingen) were cotransfected into *Spodoptera frugiperda* (Sf9) cells by the calcium phosphate method according to the manufacturer's instructions. Putative recombinant viral plaques were identified by color screening. Viruses from several putative recombinant plaques were tested by immunoblot for the ability to express recombinant X or Y domain polypeptides in infected Sf9 cells. The positive plaques were purified and the recombinant viral DNA was used to infect *Trichoplusia ni* (High-five) insect cells (Invitrogen) for large-scale production of recombinant proteins.

Insect Cell Culture. Stock cultures of Sf9 and High-five cells were grown at 27°C as monolayers in TNM-FH [Grace's insect media supplemented with lactalbumin hydrolysate and yeastolate (Invitrogen)] with 10% fetal bovine serum (Intergen, Purchase, NY), plus 10 μg/ml gentamycin (GIBCO). Confluent monolayers were subcultured by removal of cells from the flask with gentle pipetting and dilution (1:7) in TNM-FH. Production of large scale, high-titer viral stocks was performed

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Abbreviations: PLC, phospholipase C; PIP_2, phosphatidylinositol4,5-bisphosphate; PTP, phosphotyrosine phosphatase.

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in Spinner flasks (Bellco Glass) with 2×10^6 Sf9 cells/ml. Spinner cultures were stirred at 50 rpm and incubated at 27°C.

Infection of Insect Cells with Recombinant Baculoviruses. High five insect cells (2×10^7) were infected at a multiplicity of infection of 10 for 48 hr with recombinant baculovirus encoding (His_6)-X domain or (His_6)-Y domain or both simultaneously. Cells were lysed by incubation for 30 min at 4°C with a buffer containing 1% Triton X-100, 50 mM NaCl, 20 mM HEPES, pH 8.0, 10 μg of leupeptin per ml, and 10 μg of aprotinin per ml. Cell debris was removed by centrifugation ($10,000 \times g$, 10 min) at 4°C.

Affinity Purification of (His_6)-X or (His_6)-Y Domains. Purification of the fusion proteins was performed essentially as described elsewhere (14). In brief, the cell lysates were incubated with Ni^{++}-NTA-agarose (Qiagen, Chatsworth, CA) at 4°C for more than 2 hr. The resin was then washed with 10 column volumes of buffer containing: 500 mM NaCl, 20 mM Hepes (pH 8.0), 1% Triton X-100, and 10 mM imidazole. (His_6)-X domain or (His_6)-Y domain were eluted stepwise in three fractions containing two column volumes of 35, 75, or 100 mM imidazole in 500 mM NaCl, 20 mM Hepes (pH 8.0), and 1% Triton X-100. Aliquots of the fractions were analyzed by 7.5% SDS/PAGE and immunoblotting. Purified fragments were stored in elution buffer containing 20% glycerol at −80°C.

Assay of Enzyme Activity. The assay of PIP_2 hydrolysis was performed in a 50 μl reaction mixture containing the indicated concentration of [^{3}H]PIP_2 (200 μM, unless otherwise specified), 0.8 mM ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA), 1.0 mM $CaCl_2$ (free calcium 110 μM), 35 mM sodium phosphate (pH 6.8), 70 mM KCl, ≈5–20 ng purified (His_6)-X domain or (His_6)-Y domain and the varying amounts of Triton X-100. Hence, at a PIP_2 mole fraction of 0.2 the final concentrations of PIP_2 and Triton X-100 are 200 μM and 10 mM (0.05%), respectively. The reaction was incubated for 15 min at 37°C and was terminated by addition of 0.67% bovine serum albumin and 6.25% trichloroacetic acid. This time of incubation was used since control data showed that at this time point PIP_2 hydrolysis is linear. The samples were centrifuged ($16,000 \times g$, 10 min) at 4°C, and the radioactivity present in the supernatant was measured by liquid scintillation counting.

RESULTS AND DISCUSSION

Expression and Association of X and Y Domains. The association of X and Y domains in PLC isoforms may be an intrinsic property of the two domains or may require conformations induced upon translation and folding of the native protein. To determine whether X and Y domains could associate following translation as independent polypeptides, insect cells were infected with recombinant baculoviruses encoding the X polypeptide (N terminus through X domain), the Y polypeptide (Y domain through C terminus), both recombinant viruses, or virus encoding holoPLC-γ1. Extracts were prepared from each culture and precipitated with antibody to the C terminus of PLC-γ1 (19), which recognizes the Y domain polypeptide and the holoenzyme. The immunoprecipitates were then analyzed for the presence of X or Y domains by Western blotting with sequence specific N-terminal (Fig. 1*A*) or C-terminal (Fig. 1*B*) antibodies, respectively. Coprecipitation of the X domain (62 kDa) with the Y domain (47 kDa) was readily detected from lysates of cells that had been coinfected with viruses encoding X or Y domains (Fig. 1*A*, lane 3). When the amount of X domain polypeptide that coprecipitated with the Y domain was compared with the amount of unassociated X domain in the supernatant, it was

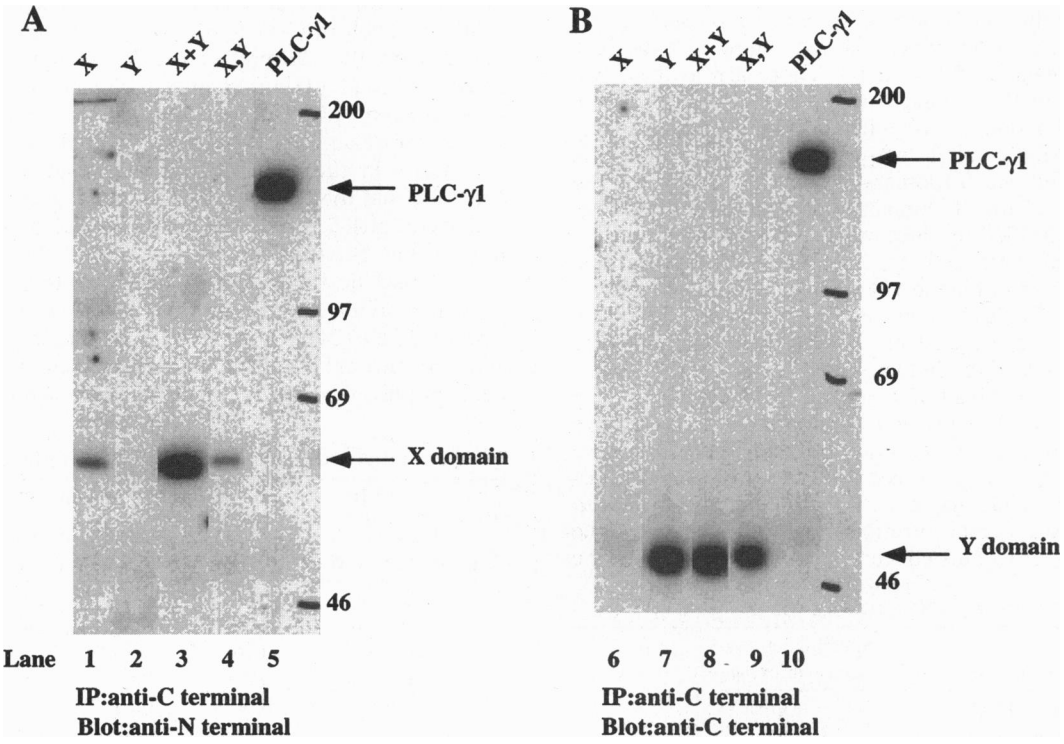

FIG. 1. Coimmunoprecipitation of (His_6)-X domain with (His_6)-Y domain. Cell lysate (500 μg) of High-five cells infected with recombinant virus encoding X or Y domains or coinfected with both viruses was incubated with a polyclonal antibody (19) to the C terminus of PLC-γ1 and protein A Sepharose for >2 hr or overnight at 4°C. The samples were washed with lysis buffer and proteins were eluted by boiling in Laemmli buffer. The samples were analyzed by 7.5% SDS/PAGE, transferred to nitrocellulose (Micron Separations, Westboro, MA), and incubated with 3% bovine serum albumin in TBST (150 mM NaCl, 50 mM Tris, pH 7.4, 0.05% Tween 20) for 1 hr at room temperature. After washing in TBST the blots were incubated with either anti-C terminal antibody or anti-N-terminal monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) for 2 hr at room temperature. The blots were washed in TBST and incubated with either ^{125}I protein A (ICN) or ^{125}I goat anti-mouse IgG (ICN) and exposed at −70°C for autoradiography.

evident that at least 50% of the total X domain was associated with the Y domain (data not shown). These results demonstrate that significant amounts of X and Y polypeptides associate *in vivo* from independently translated molecules.

Comparison of lanes 7 and 8 in Fig. 1*B* also shows that an equivalent amount of Y domain polypeptide is present in the immunoprecipitate regardless of whether the cells were infected only with virus encoding the Y domain or coinfected with X and Y domain viruses. Therefore, coexpression of the two polypeptides does not significantly influence their metabolic stability relative to expression of individual domains. Also, the molecular mass of the X and Y domain polypeptides is in agreement with the cDNA inserts employed.

To determine whether the X:Y complex could be produced *in vitro*, lysates containing the X domain polypeptide were mixed with lysates containing the Y polypeptide. Immunoprecipitation analysis (Fig. 1, lane 4) did not detect significant levels of X:Y association. When separate populations of cells expressing X and Y domains were mixed and lysed, association of the two polypeptides was not detected. Furthermore, affinity purification of the two polypeptides prior to mixing did not facilitate X and Y association.

Association of X and Y Domains Form a Functional Enzyme. To test whether the observed association of X and Y domains is functional, and thus biologically relevant, assays of enzymatic activity were performed using Triton X-100/PIP_2 mixed micelles, as described previously (20, 21). The activities of the recombinant holoenzyme, the X:Y polypeptide complex and the individual X and Y polypeptides were assayed following affinity purification (21). To compare enzyme activities, the amount of protein present in each assay was measured by Western blot analysis, as described in *Materials and Methods*, and the results presented as specific enzyme activities. The results shown in Table 1 demonstrate that when assayed individually, neither the X or Y polypeptides possesses detectable enzyme activity. However, the data show that the X:Y complex does exhibit PLC activity. There are at least two interpretations of these data. It may be that residues within both the X and Y domains directly contribute to the catalytic site. Alternatively, catalysis may require residues in the X domain only and the Y domain may be necessary for the correct folding of the X domain. Site-directed mutagenesis studies have identified residues within the X domain that are essential for catalytic activity, but similar data regarding conserved residues within the Y domain has not been reported.

The data in Table 1 show, unexpectedly, that the X:Y complex has a specific enzyme activity significantly greater (18-fold) than that of the holoenzyme. This is not due to low activity of the recombinant holoenzyme as its specific activity is comparable to that previously reported for the native enzyme isolated from bovine brain (22). Previous data have shown the specific activity of native PLC-γ1, like many phospholipid metabolizing enzymes (23), varies significantly with changes in substrate concentration expressed wither as mole fraction of PIP_2 or its bulk concentration (20). Therefore, the specific activities of holoPLC-γ1 and the X:Y complex were determined by varying both parameters of substrate availability (Table 2). The data demonstrate that the X:Y complex exhibits significantly more catalytic activity than the holoenzyme at all bulk substrate concentrations and micellar mole fractions assayed. At the highest PIP_2 concentration tested (400 μM, 0.2% mole fraction) the activities of both the holoenzyme and the X:Y complex were maximal and the activity of the X:Y complex was 17-fold higher than that of holoPLC-γ1. At the lowest substrate concentration tested (100 μM and 0.01% mole fraction) the X:Y complex exhibited a specific activity 113-fold greater than that of holoPLC-γ1. The specific activity of the holoenzyme varied ≈15-fold between the lowest and highest substrate concentrations, while the activity of the X:Y complex varied only 2.3-fold. These results indicate that the activity of the X:Y complex is considerably more resistant to reduced substrate concentration than is the activity of the holoenzyme. The activities of both holoPLC-γ1 and the X:Y complex were completely dependent on the presence of Ca^{2+} (data not shown).

Since holoPLC-γ1 contains a central SH region and the X:Y complex does not, these results support the idea that the SH region may function as an intrinsic negative regulator of basal activity of the holoenzyme. An intramolecular role for SH domains in the regulation of basal enzyme activity also has been suggested by studies of the phosphotyrosine phosphatases (PTP)1 (24) and PTP2 (25). Deletion of the two N-terminal SH2 domains in PTP1 (26) and PTP2 activates these enzymes (25). The proposed intramolecular interaction of SH2 domains with the PTP1 catalytic domain was also reflected in distinct pH optima for the PTP1 holoenzyme (pH 6.3) vs. the PTP1 deletion mutant lacking SH2 domains (pH 5.6) (27). Therefore, we have compared the enzyme activity pH profiles of holoPLC-γ1 and the X:Y complex. The data in Fig. 2 show that activity of the X:Y complex is detected across a broad pH range (6.0–7.5), with a maximum at approximately pH 7.2. In contrast, the holoenzyme has a sharp peak of activity at approximately pH 5.0 and a shoulder of activity in the physiological pH range of 6.5–7.5. We conclude that the catalytic domain per se exhibits maximum activity at pH 7.2. For the holoenzyme, however, charge interactions between the SH region and the catalytic X:Y regions may be influenced by pH and, thereby, modulate enzyme activity. A similar model has been presented in the case of PTP1 (24). Thus, SH regions may regulate enzyme activities through an intramolecular mechanism not dependent on phosphotyrosine or proline-rich sequences.

Table 1. *In vitro* phospholipase C activity

PLC-γ1	Specific activity, μmol/min/mg
Holo PLC-γ1	1.8
X + Y	32.0
X	ND
Y	ND

Approximately 40 ng of Ni^{++} affinity-purified holoPLC-γ1 or 5 ng each X or Y domain polypeptides were used per assay to measure hydrolysis of 200 μM [^{3}H]PIP_2 as described. To quantitate the X and Y domain polypeptides by immunoblotting and scanning by a PhosphorImager, a standard curve of known amounts of holoPLC-γ1 was included in each experiment. ND, not detectable.

Table 2. Influence of substrate availability on PLC activity *in vitro*

PIP_2		Enzyme		
Bulk concentration, μM	Mole %	Holo PLC-γ1	X:Y complex	Fold increase
100	0.01	0.2	22.5	113
100	0.05	0.3	18.2	61
100	0.10	0.3	23.3	78
100	0.20	0.8	23.6	30
200	0.01	0.2	12.0	60
200	0.05	0.8	23.1	29
200	0.10	0.8	30.0	37
400	0.01	0.5	26.4	53
400	0.05	0.7	41.5	59
400	0.20	3.0	51.3	17

The effect of varying the substrate concentration on the specific activities (μmol/min/mg) of holoPLC-γ1 and X:Y complex was measured by changing bulk PIP_2 concentration or by changing the mole fraction of PIP_2 in the Triton X-100 micelle as described.

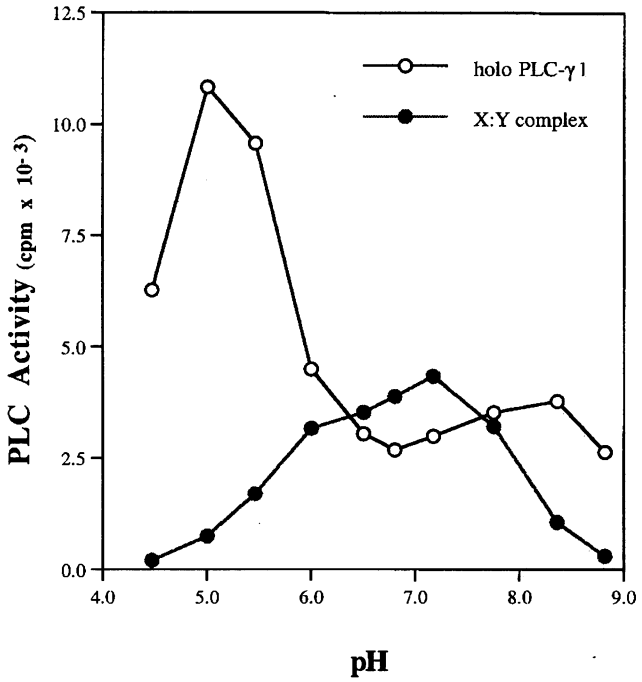

FIG. 2. Influence of pH on the enzyme activities of holoPLC-γ1 and X:Y complex. Approximately 50 ng of purified holoPLC-γ1 or 10 ng of purified X:Y complex were incubated with 200 μM [^{3}H]PIP_2, 70 mM KCl, 10 mM Triton X-100, and 50 mM Tris acetate at the indicated pH values for 15 min at 37°C. Reactions were terminated as described. Similar pH activity profiles were obtained for holoPLC-γ1 in assays performed with 50 mM Mes (pH 4.0–6.0), 50 mM sodium citrate (pH 4.0–6.0), 35 mM sodium phosphate (pH 6.0–8.0) or 20 mM Hepes (pH 6.0–8.0).

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1. Abdel-Latif, A. A. (1986) *Pharmacol. Rev.* **38,** 227–272.
2. Bristol, A., Hall, S. M., Kriz, R. W., Stahl, M. L., Fan, Y. S., Byers, M. G., Eddy, R. L., Shows, T. B. & Knopf, J. L. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **58,** 915–920.
3. Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nadanishi, O., Suzuki, K. & Takenawa, T. (1989) *J. Biol. Chem.* **264,** 21885–21890.
4. Ellis, M. V., Carne, A. & Katan, M. (1993) *Eur. J. Biochem.* **213,** 339–347.
5. Cifuentes, M., Honkanen, L. & Rebecchi, M. J. (1993) *J. Biol. Chem.* **268.**
6. Ellis, M. V., U., S. & Katan, M. (1995) *Biochem. J.* **307,** 69–75.
7. Fernald, A. W., Jones, G. A. & Carpenter, G. (1994) *Biochem. J.* **302,** 503–509.
8. Rhee, S. G. (1991) *Trends Biochem. Sci.* **16,** 297–301.
9. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C. & Pawson, T. (1991) *Science* **252,** 668–674.
10. Rönnstrand, L., Mori, S., Arridsson, A. K., Eriksson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L. & Heldin, C. H. (1992) *EMBO J.* **11,** 3911–3919.
11. Seedorf, K., Kostka, G., Lammers, R., Bashkin, P., Daly, R., Burgess, W. H., vanderBliek, A. M., Schlessinger, J. & Ullrich, A. (1994) *J. Biol. Chem.* **269,** 16009–16014.
12. Scaife, R., Gout, I., Waterfield, M. D. & Margolis, R. L. (1994) *EMBO J.* **13,** 2574–2582.
13. Soler, C., Beguinot, L., Sorkin, A. & Carpenter, G. (1993) *J. Biol. Chem.* **268,** 22010–22019.
14. Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. & Carpenter, G. (1990) *Science* **250,** 1253–1256.
15. Koblan, K. S., Schaber, M. D., Edwards, G., Gibbs, J. B. & Pompliano, D. L. (1995) *Biochem. J.* **305,** 745–751.
16. Homma, Y., Emori, Y. & Takenawa, T. (1992) *J. Biol. Chem.* **267,** 3778–3782.
17. Homma, Y., Emori, Y. & Takenawa, T. (1992) *Biochem. Biophys. Res. Comm.* **182,** 1402–1407.
18. Homma, Y. & Takenawa, T. (1992) *J. Biol. Chem.* **267,** 21844–21849.
19. Arteaga, C. L., Johnson, M. D., Todderud, G., Coffey, R. J., Carpenter, G. & Page, D. L. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10435–10439.
20. Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G. & Carpenter, G. (1992) *J. Biol. Chem.* **267,** 10447–10456.
21. Horstman, D. A. & Carpenter, G. (1995) *Prot. Expression Purif.* **6,** 278–283.
22. Ryu, S. H., Cho, K. S., Lee, K.-Y., Suh, P.-G. & Rhee, S. G. (1987) *J. Biol. Chem.* **262,** 12511–12518.
23. Carman, G. M., Deems, R. A. & Dennis, E. A. (1995) *J. Biol. Chem.* **270,** 18711–18714.
24. Pei, D., Lorenz, U., Klingmuller, U., Neel, B. G. & Walsh, C. T. (1994) *Biochemistry* **33,** 15483–15493.
25. Zhao, Z., Larocque, R., Ho, W.-T., Fischer, E. H. & Shen, S.-H. (1994) *J. Biol. Chem.* **269,** 8780–8785.
26. Townley, R., Shen, S.-H., Banville, D. & Ramachandran, C. (1993) *Biochemistry* **32,** 13414–13418.
27. Pei, D., Neel, B. G. & Walsh, C. T. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1092–1096.