Limited proteolysis of phospholipase C- γ 1 indicates stable association of X and Y domains with enhanced catalytic activity

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Phospholipase C- γ 1 (PLC- γ 1) was treated with *Staphylococcus aureus* V8 protease (V8) and the digestion products were analysed with site-specific antibodies. V8 treatment generated three immunodetectable PLC- γ 1 fragments of 120, 97, and 39 kDa. The 39 kDa fragment is derived from the C-terminus of PLC- γ 1 and includes the conserved Y domain present in all PLC isoenzymes. The 120 and 97 kDa fragments are derived from the N-terminus of PLC- γ 1, possess the conserved X domain common to all PLC isoenzymes, and the *src*-homology domains unique to PLC- γ 1 and - γ 2. It is likely that the 97 kDa fragment is a V8 product of the 120 kDa fragment. As the C-terminal 39 kDa

fragment, and either of the N-terminal 120 or 97 kDa fragments, were precipitable with antibody specific to a sequence present in only the 39 kDa fragment, the data indicate co-precipitation of separate polypeptide chains that remain associated after V8 proteolysis. Importantly, V8 treatment increased the activity of PLC- γ 1 and did not alter the calcium requirement. The influence of other modulators of PLC- γ 1 activity, however, was lost following V8 treatment. These results suggest the stable association of the X and Y domains within PLC- γ 1, and demonstrate that proteolysis in the region of PLC- γ 1 that is subject to tyrosine phosphorylation can enhance catalytic activity.

INTRODUCTION

Phospholipase C (PLC) isoenzymes hydrolyse PtdIns(4,5) P_2 producing two second-messenger metabolites, Ins(1,4,5) P_3 and diacylglycerol (Rhee et al., 1989). Based on amino acid sequence, PLC isoenzymes are grouped into three families: PLC β (β 1–4), PLC γ (γ 1 and 2), and PLC δ (δ 1–3) (Rhee et al., 1989). Activity of PLC β isoenzymes is regulated by heterotrimeric GTP-binding proteins (Blank et al., 1991; Smercka et al., 1991; Taylor et al., 1991; Waldo et al., 1991), while the activity of PLC γ isoenzymes is controlled by tyrosine phosphorylation (Nishibe et al., 1990; Kim et al., 1990). The mechanism of PLC δ regulation remains unclear.

All PLC isoenzymes share two regions of sequence similarity designated X and Y domains (Rhee et al., 1989). It is probable that these conserved regions are part of a catalytic site, as point mutations or deletions in either domain abolish enzyme activity (Bristol et al., 1988; Emori et al., 1989; Ellis et al., 1993; Wu et al., 1993). Proteolysis studies with PLC- δ indicate that the X and Y domains remain physically associated after trypsin cleavage between these domains (Cifuentes et al., 1993; Ellis et al., 1993). However, these two studies reported different results as to the effect of trypsin proteolysis on PLC- δ activity.

PLC- γ 1 and - γ 2 differ from other PLC isoenzymes in that a 400-residue region, containing two SH2 domains and one SH3 domain, is located between the X and Y domains (Stahl et al., 1988; Suh et al., 1988). The SH2 and SH3 domains of PLC- γ are not obligatory for enzyme activity, as the enzyme retains significant activity when the SH2/SH3 region is deleted (Bristol et al., 1988; Emori et al., 1989). The SH2 domains of PLC- γ 1 are involved, however, in the phosphotyrosine-dependent association of PLC- γ 1 with activated growth-factor receptors (Anderson et al., 1990; Margolis et al., 1990a,b; Mohammadi et al., 1991; Rotin et al., 1992; Soler et al., 1993). This interaction is thought to be a prerequisite for the phosphorylation of PLC- γ 1 tyrosine

residues by activated receptor tyrosine kinases (Mohammadi et al., 1992; Peters et al., 1992; Ronnstrand et al., 1992; Soler et al., 1993; Valius et al., 1993). The SH3 domain is thought to function by facilitating the association of PLC- γ 1 with other proteins (Bar-Sagi et al., 1993; Gout et al., 1993).

As there is no information regarding higher-order structure–function relationships in PLC- γ 1, we have utilized limited protease digestion to study domain interactions and enzymic activity in PLC- γ 1.

MATERIALS AND METHODS

Cell culture

A-431 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% (v/v) calf serum (GIBCO) and 50 μ g/ml gentamicin (Sigma). A-431 cells were subcultured at a density of $(4\text{--}8) \times 10^6$ cells per 245 mm² culture dish (USA Scientific Products) and grown until 70–100% confluent [approx. $(6\text{--}9) \times 10^7$ cells].

Preparation of cytosol

A-431 cells were washed three times at 37 °C with DMEM and incubated in DMEM at 37 °C for 30 min. When the cells were used to prepare phosphorylated PLC- γ 1, 500 ng/ml epidermal growth factor (EGF) (Savage and Cohen, 1972) was added for 10 min at 37 °C. In all experiments, culture dishes were rapidly washed four times with cold Ca²⁺-, Mg²⁺-free PBS and the cells were scraped into 250 ml of cold homogenization buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 10% glycerol, 50 mM β -glycerophosphate, 0.2 mM Na₃VO₄, 1 mM EGTA) per plate. The cells were then lysed with 20 strokes of a rapidly spinning Teflon homogenizer (Wheaton Scientific). The lysates were centrifuged (174000 *g*, 20 min) at 4 °C and supernatants recovered as the cytosol fraction. The protein concentration of

Abbreviations used: PLC- γ 1, phospholipase C- γ 1; PLC- δ 1, phospholipase C- δ 1; EGF, epidermal growth factor; SH, *src* homology; V8, *Staphylococcus aureus* V8 protease; DMEM, Dulbecco's modified Eagle's medium.

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the cytosol was determined utilizing a bicinchoninic acid protein assay (Pierce).

Limited proteolysis of PLC- γ 1

Immobilized *Staphylococcus aureus* V8 protease (V8) (Pierce) was pre-washed once with homogenization buffer to remove free enzyme. A-431 cytosol, in an Eppendorf tube, was incubated on a rocker with immobilized V8 (5–15 μ l of a 55% slurry/100 μ g of cytosol protein) for 30 min at room temperature. V8 digestion was stopped by puncturing a hole in the bottom of the tube and centrifuging (5000 g , 3–5 min). The eluate was collected in a screw-top 1.5 ml capacity tube as the digested cytosol, while the V8-agarose beads were retained in the Eppendorf tube.

Immunoprecipitation and Immunoblotting

To immunoprecipitate PLC- γ 1, equal amounts (400 μ g) of A-431 cytosol (control or V8-treated) were incubated with antisera (anti-C-terminus antibody) prepared against residues 1249–1262 near the C-terminus of PLC- γ 1 (Arteaga et al., 1991). Approx. 1 μ l of the antisera was used for each 100 μ g of cytosolic protein. At the same time 10 μ l of 10% (v/v) heat-inactivated *S. aureus* cells (Calbiochem) per 1 μ l of antibody was also added. Immunoprecipitation occurred overnight at 4 °C. PLC- γ 1 immunoprecipitates were collected by centrifugation, washed two or three times with RIPA buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.02% NaN_3) at 37 °C, solubilized in Laemmli buffer (Laemmli, 1970), and heated at 100 °C for 5 min. The samples were then centrifuged (5000 g , 5 min) and subjected to electrophoresis on an SDS/10% polyacrylamide gel (Laemmli, 1970).

After electrophoresis, proteins were transferred to nitrocellulose for immunoblotting in a chamber (Bio-Rad) containing transfer buffer [50 mM Tris, 380 mM glycine, 20% (v/v) methanol] at 100 mA for 1–2 h at 4 °C. Before immunoblotting, the nitrocellulose was first incubated in blocking solution of 5% (w/v) non-fat dry milk (for polyclonal antibodies) or 1% (w/v) BSA (for monoclonal antibodies) in a Tris-buffered saline (15 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween 20) solution for 1 h at room temperature, or overnight at 4 °C. The nitrocellulose was then incubated for 2 h in Tris-buffered saline with antisera (1:500) to PLC- γ 1 [anti-(C-terminus, residues 1249–1620) antibody], or a monoclonal antibody (1:1000) to PLC- γ 1 [anti-(N-terminal, residues 81–100) antibody] (Transduction Laboratories), or a polyclonal anti-phosphotyrosine antibody (1:2000) (Zymed Laboratories), or a monoclonal antibody (1:500) to the SH3 domain of PLC- γ 1 (Park and Rhee, 1992). Bound polyclonal antibodies were detected by autoradiography using ^{125}I -labelled Protein A (ICN), while monoclonal antibodies were detected with goat antibodies raised to mouse immunoglobulin G conjugated with horseradish peroxidase (Cappel), followed by chemiluminescence (DuPont). Blots were developed using an X-OMAT Processor (Kodak) after exposure to X-OMAT film (Kodak). Radioactive or chemiluminescence signals respectively, were quantified using a PhosphorImager (Molecular Dynamics) or an EC910 scanning densitometer (E-C Apparatus Corporation) in conjunction with GS-370 data-systems software (Hoefer Scientific).

Metabolic radiolabelling of A-431 cells

A-431 cells were subcultured at a density of 5×10^5 cells per 100-mm-diam. culture dish (Costar). When the cells reached approx.

70% confluency (approx. 4×10^6 cells), the cultures were incubated with 50 $\mu\text{Ci/ml}$ [^{35}S]methionine-cysteine-TransLabel (ICN) in DMEM plus 10% (v/v) calf serum, for 20 h. The cultures were then washed four times with cold Ca^{2+} -, Mg^{2+} -free PBS, solubilized by adding 1 ml of solubilization buffer [1% (v/v) Triton-X-100, 20 mM Hepes, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 50 mM β -glycerophosphate, 0.2 mM Na_3VO_4] and incubated on a shaker for 10–20 min at 4 °C. The radio-labelled extracts were clarified by centrifugation (12000 g , 5 min) before treatment with immobilized V8 as described above.

To immunoprecipitate PLC- γ 1 from the radiolabelled extracts, equal amounts of protein from control and V8-treated extracts were incubated with the anti-(C-terminal PLC- γ 1) antibody (1 μ l/100 μ g of protein) and Protein A–Sepharose CL-4B (Sigma) [10 μ l of a 20% (w/v) slurry/1 μ l of antibody] overnight at 4 °C. The immunoprecipitates were washed and subjected to SDS/PAGE as described above. The polyacrylamide gel was incubated in a fixing solution [30% (v/v) methanol, 10% (v/v) acetic acid] for 30 min, treated with En 3 Hance (DuPont) for 15–20 min, and dried. Radioactive protein in the dried gel was detected by fluorography.

PLG- γ 1-activity measurement

To prepare immunoprecipitated PLC- γ 1 for enzyme activity measurement, control and V8-treated cytosols were incubated with the anti-(C-terminal) antibody as described above. Immunoprecipitates were washed three times with ice-cold homogenization buffer instead of RIPA buffer. Substrate, $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ (DuPont–NEN) and $\text{PtdIns}(4,5)\text{P}_2$ ammonium salt (Boehringer–Mannheim), preparation and the measurement of $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ hydrolysis were as described by Wahl et al. (1992). The hydrolysis of $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ was measured in a 50 μ l total reaction volume that contained 35 mM NaH_2PO_4 , pH 6.8, 70 mM KCl, 0.8 mM EGTA, 0.8 mM CaCl_2 , 200 μM $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ (approx. 2 c.p.m./pmol), 1.8 mM Triton X-100, and PLC- γ 1 was immunoprecipitated from 100 μ g of control or V8-treated cytosol as described above. Unless otherwise indicated, assays were performed at 200 μM $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ (mol fraction $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ of 0.1). Substrate hydrolysis was initiated by adding a 10 μ l aliquot of the immunoprecipitated PLC- γ 1 suspension to 40 μ l of substrate solution. Duplicate reactions were incubated at 37 °C for 15 min unless otherwise indicated and terminated as previously described (Wahl et al., 1992).

RESULTS

Immunodetection of PLC- γ 1 fragments following V8 treatment

To determine whether PLC- γ 1 was digested by V8 protease, A-431 cytosol was treated with V8, and, after removal of the protease, incubated with anti-(C-terminal PLC- γ 1) antibody. The immunoprecipitates were then electrophoresed and blotted with either antibodies to the C- or N-termini of PLC- γ 1. The results in Figure 1(a) show that increasing the amounts of V8 present results in the loss of native PLC- γ 1 (145 kDa) and the formation of a 39 kDa fragment of PLC- γ 1 recognized by the anti-(C-terminus) antibody. Based on apparent molecular mass and the recognition site of the anti-(C-terminus) antibody, the 39 kDa fragment probably encompasses the Y domain of PLC- γ 1, with the site of proteolysis at an acidic residue between the SH3 domain and Y domain of PLC- γ 1 (see Figure 6a).

The results in Figure 1(b) show that two additional V8 fragments (97 kDa and 120 kDa) of PLC- γ 1, recognized by an anti-(N-terminus) antibody to PLC- γ 1, are present also in

Figure 1 Immunodetection of PLC- γ 1 fragments after V8 digestion

A-431 cytosol (400 μ g per sample) was incubated with the indicated amounts of immobilized V8 per 100 μ g of cytosolic protein for 30 min at room temperature. After removal of V8 by centrifugation, each sample was incubated with the anti-(C-terminus) antibody to PLC- γ 1. Immunoprecipitates were then electrophoresed and immunoblotted as described in the Materials and methods section. (a) Blot with anti-(C-terminus) antibody to PLC- γ 1. (b) Blot with anti-(N-terminus) antibody to PLC- γ 1.

immunoprecipitates generated by the anti-(C-terminus) antibody. As neither of these fragments was detected by the anti-(C-terminus) antibody, it is concluded that the 120 kDa and 97 kDa fragments are physically associated with the 39 kDa fragment. Treatment of immunoprecipitated V8-treated PLC- γ 1 with 30 mM 2-mercaptoethanol, or 1.0 M NaCl, or 750 mM potassium thiocyanate did not disrupt the association of the C- and N-terminal PLC- γ 1 fragments (results not shown).

Based on apparent molecular masses and the anti-(N-terminus)-antibody recognition sites, the 120 kDa and 97 kDa fragments encompass the N-terminal region of PLC- γ 1 and the X domain. As discussed below, the 97 kDa fragment appears to be a proteolytic product of the 120 kDa fragment.

Properties of V8-generated fragments of PLC- γ 1

To distinguish whether the V8 fragments encompass the SH3 domain of PLC- γ 1, proteolytic products were isolated by immunoprecipitation with the anti-(C-terminus) antibody, electrophoresed, and blotted with an antibody against the SH3 domain of PLC- γ 1 (Park and Rhee, 1992). While native PLC- γ 1 and the 120 kDa V8-treated fragment were clearly recognized by

Figure 2 Properties of V8 fragments of PLC- γ 1

A-431 cytosol (400 μ g per sample) was incubated with the indicated amounts of immobilized V8 per 100 μ g of cytosolic protein for 30 min at room temperature. After removal of V8 by centrifugation, each sample was incubated with the anti-(C-terminus) antibody to PLC- γ 1, and the immunoprecipitates were electrophoresed and immunoblotted as described in the Materials and methods section. (a) Blot with antibody to the SH3 domain of PLC- γ 1. (b) Blot with anti-phosphotyrosine antibody.

the SH3 antibody, neither the 97 kDa nor the 39 kDa fragment was detected (Figure 2a). Therefore, the site of V8 proteolysis that generates the 97 kDa fragment must occur between the SH2 and SH3 domains, while the site of proteolysis for the 39 kDa fragment must be after the SH3 domain (see Figure 6a).

In the native PLC- γ 1 molecule, the EGF receptor produces tyrosine phosphorylation at Tyr-771 and Tyr-783, which are located between the SH2 and SH3 domains, and at Tyr-1254, located near the C-terminus (Kim et al., 1990; Wahl et al., 1990). To identify which V8 proteolytic products of PLC- γ 1 possess tyrosine phosphorylation sites, A-431 cells were treated with EGF for 10 min at 37 $^{\circ}$ C to maximally phosphorylate PLC- γ 1. Equal amounts of cytosol from control and EGF-treated cells were treated with V8 and then incubated with the anti-(C-terminus) antibody to PLC- γ 1, electrophoresed and blotted with antibody to phosphotyrosine. All three V8-treated fragments derived from PLC- γ 1 were recognized by the anti-phosphotyrosine antibody (Figure 2b), although the 97 kDa fragment was considerably reduced in anti-phosphotyrosine reactivity relative to the 120 kDa and 39 kDa fragments.

V8 proteolysis of metabolically labelled PLC- γ 1 immunoprecipitates

To determine whether the fragments detected immunologically are an accurate representation of V8 fragments of PLC- γ 1, A-431 cells were metabolically labelled with [35 S]methionine. 35 S labelling also permits an assessment of the relative abundance of individual V8 proteolysis fragments that is not possible with comparisons between different antibodies. 35 S-labelled cytosol was treated with V8 and PLC- γ 1 was immunoprecipitated with the anti-(C-terminus) antibody. This analysis could only be performed following precipitation with the anti-(C-terminus) antibody to PLC- γ 1, as the anti-(N-terminal) antibody is not a precipitating antibody.

The results in Figure 3 show that three 35 S-labelled fragments of 120 kDa, 97 kDa and 39 kDa were detected. After V8 treatment, the total amount of 35 S-labelled V8 fragments was 37 % (lane 2), 34 % (lane 3), and 23 % (lane 4) of the initial amount of intact PLC- γ 1 (lane 1). Metabolic labelling, therefore, does not show the presence of major V8-cleaved fragments not previously recognized by antibody detection. However, the low recovery of fragments relative to the initial amount of PLC- γ 1 suggests that either additional site(s) of V8 proteolysis occur in the C-terminal region of PLC- γ 1, which prevent immunoprecipitation by the anti-(C-terminus) antibody, or that the observed fragments are subject to further proteolysis and do not therefore accumulate quantitatively relative to intact PLC- γ 1. Based on apparent molecular mass and 35 S content, the average relative abundances (Figure 3, lanes 2–4) of the three fragments are 17 % (120 kDa), 32 % (97 kDa) and 51 % (39 kDa). Thus there is an approximate 1:1 ratio of the 39 kDa fragment to both the 120 kDa and 97 kDa fragments.

Influence of V8 treatment on PLC- γ 1 activity

To demonstrate further whether the V8 proteolytic fragments of PLC- γ 1 retain enzymic activity, intact and V8-treated PLC- γ 1 were immunoprecipitated from A-431 cytosols with anti-(C-terminal PLC- γ 1) antibody. An aliquot of the washed immunoprecipitate was removed and PLC- γ 1 activity was measured as described in the Materials and methods section. The results shown in Figure 4 demonstrate that limited V8 proteolysis of PLC- γ 1 resulted in an apparent increase (approx. 2-fold) in enzyme activity. In this experiment the amount of intact PLC- γ 1 remaining after V8 treatment is shown in Figure 1(b), which is part of the same experiment. Quantification indicates that treatment with 5, 10, or 15 μ l of V8 reduced the amount of intact PLC- γ 1 respectively to 13.6 %, 0.8 %, and 0 % of the untreated control. Therefore, enzymic activity was retained by the V8 fragments of PLC- γ 1 in the absence of any detectable intact enzyme.

Influence of modulators of PLC- γ 1 activity following V8 treatment

PLC- γ 1 requires Ca^{2+} for enzyme activity (Ryu et al., 1987). To show whether the proteolytic fragments of PLC- γ 1 retain this Ca^{2+} requirement, the activities of the PLC- γ 1 fragments were assayed in the absence or presence of free Ca^{2+} (Table 1, experiment 1). The results show that both intact and V8-treated PLC- γ 1 behaved similarly, as enzyme activity was lost in the absence of free Ca^{2+} . AMP has been shown to inhibit PLC- γ 1 activity (S. G. Rhee, personal communication). To determine whether AMP inhibited the activity of the PLC- γ 1 V8 fragments, native and V8-treated PLC- γ 1 were incubated in the absence and presence of 1 mM AMP for 30 min at 4 °C and then assayed for

Figure 3 Analysis of 35 S-labelled V8 fragments of PLC- γ 1

A-431 cells were incubated for 20 h with [35 S]Translabel and cell extracts were prepared as described in the Materials and methods section. The extracts (800 μ g per sample) were then treated with the indicated amounts of immobilized V8 per 100 μ g of cytosolic protein. After removal of V8 by centrifugation, the samples were incubated with the anti-(C-terminus) antibody to PLC- γ 1. The immunoprecipitates were then electrophoresed and fluorography performed. Radioactivity in each band was quantified by scanning the films with a PhosphorImager.

Figure 4 Influence of V8 treatment on PLC- γ 1 activity

A-431 cytosols (700 μ g per sample) were incubated with the indicated amount of immobilized V8 per 100 μ g of cytosolic protein for 30 min at room temperature. After removal of V8 by centrifugation, each sample was incubated with the anti-(C-terminus) antibody to PLC- γ 1. Immunoprecipitated samples were then resuspended in homogenization buffer and assayed for PLC activity or immunoreactivity. Data are the averages of five separate experiments.

enzyme activity (Table 1, experiment 2). While intact PLC- γ 1 was inhibited 58 % by AMP, the activity exhibited by the V8 fragments was not decreased. Phosphatidic acid increases PLC- γ 1 activity by acting as an allosteric modifier (Jones and

Table 1 Sensitivity of V8-treated PLC- γ 1 to modulators of PLC- γ 1 activity

In each experiment, A-431 cytosol (900 μ g) was incubated with each of the indicated amounts of V8 per 100 μ g of cytosolic protein for 30 min at room temperature. After removal of V8 by centrifugation, each sample was incubated with the anti-(C-terminus) antibody to PLC- γ 1. Immunoprecipitated samples were then resuspended in homogenization buffer and assayed for PLC activity. In the first experiment, aliquots of the control and V8-treated samples were assayed for PLC activity in the absence or presence of free Ca^{2+} . In the second experiment, control and V8-treated samples were preincubated in the absence or presence of AMP for 30 min at 4 $^{\circ}\text{C}$ and an aliquot of each was then assayed for PLC- γ 1 activity. In the third experiment, aliquots of control and V8-treated samples were assayed for PLC- γ 1 activity in the absence or presence of phosphatidic acid (PA). The PLC- γ 1 assays containing phosphatidic acid were terminated after a 5 min incubation. Each experiment was performed at least twice.

Addition/deletion to assay	V8(μ l) ...	PLC- γ 1 activity (pmol of $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ hydrolysed/min)					
		0	5	10	15	20	40
Experiment 1							
– Ca^{2+}		0.0	0.4	0.2	0.0	–	–
+ Ca^{2+} (1 μM)		9	25	33	35	–	–
Experiment 2							
– AMP		15	33	31	23	–	–
+ AMP (1 mM)		7	29	27	25	–	–
Experiment 3							
– PA		28	–	38	–	20	11
+ PA (500 μM , 0.2 mol fraction)		256	–	102	–	40	14

Figure 5 Influence of increasing mol fraction of substrate on the activity of V8-treated PLC- γ 1

A-431 cytosol (1.5 mg-per sample) was treated with or without 15 μ l of V8 per 100 μ g of cytosolic protein. After removal of V8 by centrifugation, each sample was incubated with the anti-(C-terminus) antibody to PLC- γ 1. Aliquots of control (■) and V8-treated (□) immunoprecipitates were assayed for PLC- γ 1 activity at the indicated mol fractions with a total substrate concentration of 200 μM .

Carpenter, 1993). To measure the influence of phosphatidic acid on enzyme activity exhibited by the V8 fragments of PLC- γ 1, native and V8-treated PLC- γ 1 were assayed for PLC activity in

the absence and presence of phosphatidic acid (Table 1, experiment 3). The results demonstrate that phosphatidic acid increased (approx. 9-fold) the activity of intact PLC- γ 1. However, following V8 treatment, the ability of the fragmented enzyme to be modulated by phosphatidic acid was lost.

Immunodetection with antibodies raised against either the C- or N-terminal of PLC- γ 1 was performed to confirm that proteolysis of PLC- γ 1 occurred in each of the V8-treatment conditions described in Table 1. In all samples treated with 10 μ l or more of V8, the native 145 kDa form of PLC- γ 1 was not detectable (results not shown).

Influence of increasing substrate concentrations on the activity of V8-fragmented PLC- γ 1

As co-operativity is a characteristic of native PLC- γ 1 (Wahl et al., 1992), the influence of increasing the mol fraction of substrate on the enzymic activity exhibited by V8 fragments was evaluated (Figure 5). Intact and V8-treated PLC- γ 1 were prepared by immunoprecipitation and assayed for PLC activity at $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ mol fractions ranging from 0.05 to 0.30. The data show that intact PLC- γ 1 exhibited co-operativity as previously reported (Wahl et al., 1992). In contrast, the V8-treated enzyme produced a biphasic curve. At mol fractions below 0.1, the PLC activity of the V8 fragments was increased as much as 20-fold relative to the native enzyme. As the mol fraction of substrate increased to 0.2, however, the activity of the V8-treated enzyme decreased to approx. 50% that of the native enzyme. The enzyme activity of the V8-treated PLC- γ 1 subsequently increased as the mol fraction of $\text{Ptd}[\text{^3H}]\text{Ins}(4,5)\text{P}_2$ increased to 0.3. At this point the PLC activity of V8-treated enzyme was 75% that of the activity of native enzyme. In this experiment, there was no immunodetectable intact PLC- γ 1 in the V8-treated sample (results not shown). In each of four repeats of this experiment, a biphasic profile of activity was exhibited by the V8-fragmented enzyme.

DISCUSSION

The data in this manuscript show that limited V8 treatment of PLC- γ 1 cleaves the enzyme at sites producing three detectable fragments, as depicted in Figure 6(a). The exact sites of V8 proteolysis could not be determined as insufficient amounts of protein were available for sequencing. Based on the reactivity of site-specific antibodies, however, we conclude that primary V8 cleavage of PLC- γ 1 occurs within the 10 acidic residues of residues 910–952, which are located between the SH3 and Y domains. V8 cleavage at this site produces an N-terminal (120 kDa) and a C-terminal (39 kDa) fragment. An N-terminal 97 kDa fragment appears to be generated by V8 cleavage, probably of the 120 kDa fragment, at either Glu-776 or Glu-785, as these residues are the only two potential V8 cleavage sites between Tyr-771 and the SH3 domain. This is consistent with immunological analysis showing that the 97 kDa fragment does contain phosphotyrosine (Figure 2b), but not the SH3 domain (Figure 2a). As the 97 kDa fragment contains less phosphotyrosine than the 120 kDa fragment (Figure 2b), but accumulates to the same abundance as the 120 kDa fragment (Figure 3), this suggests that the V8 cleavage site may be Glu-776 which would eliminate a main phosphorylation site at Tyr-783 from the 97 kDa fragment.

Both co-precipitation and density-gradient analyses (results not shown) of V8-treated PLC- γ 1 indicate that the Y domain (39 kDa fragment) and X domain (120 or 97 kDa fragments) are physically associated and exhibit PLC activity. This observation

Figure 6 Schematic illustration of V8 proteolysis of PLC- γ 1

(a) Diagram of fragments produced by V8 proteolysis of PLC- γ 1. (b) A schematic illustration of putative domain interactions and putative V8 hydrolysis sites in PLC- γ 1.

is consistent with the interpretation that these conserved regions, common to all PLC isoenzymes, constitute the catalytic site of PLC isoenzymes (Rhee et al., 1989). The data on PLC- γ 1 and PLC- δ 1 suggest a common tertiary structural organization among the PLC isoenzymes.

Others have presented evidence that PLC- δ 1 is organized such that the X and Y domains interact after trypsin proteolysis between these two domains (Cifuentes et al., 1993; Ellis et al., 1993). In regard to enzymic activity after protease treatment of PLC- δ 1, Cifuentes et al. (1993) demonstrated that trypsin also cleaved 60 N-terminal residues in PLC- δ 1 and this produced a 90% decrease in enzyme activity. However, Ellis et al. (1993) did not observe trypsin proteolysis in the N-terminal region of PLC- δ 1 and reported that PLC- δ 1 activity is slightly (approx. 20%) increased after trypsin proteolysis. These authors also showed that mutagenic deletion of 135 N-terminal residues of PLC- δ 1 did not affect enzyme activity. It should be noted that the differing results of these two studies in regard to the enzyme activity of trypsin-treated PLC- δ 1 may be attributed, in part, to different methods used to assay enzymic activity.

The immunoprecipitated complex of PLC- γ 1 V8-cleaved fragments retains enzymic activity and, in fact, is apparently increased approx. 2-fold (Figure 4). However, quantitative analysis of ^{35}S -labelled digestion products (Figure 3) shows that V8 digestion results in the loss of approx. 75% of the total immunoprecipitable PLC- γ 1. Therefore, if the activity data are corrected for the relative amounts of enzyme in the control and

V8-treated samples, it appears that PLC- γ 1 specific activity is actually increased by as much as 4-fold following V8 proteolysis. Kinetic analysis (Figure 5) shows that this apparent activation of PLC- γ 1 by V8 is particularly high at low mol fractions of Ptd[^3H]Ins(4,5) P_2 , conditions which limit activity of the control enzyme (Wahl et al., 1992) and which may represent a more physiological level of this phospholipid. This result implies that V8 cleavage of PLC- γ 1 overcomes the co-operativity displayed by the native enzyme (Wahl et al., 1992). Homma and Takenawa (1992) have reported data consistent with a model in which a PLC inhibitory region, located between the SH2 and SH3 domains, reduces PLC- γ 1 catalytic activity by interacting with the putative catalytic domain(s). It is possible that V8 proteolysis of PLC- γ 1 in this same region disrupts the inhibitory region. Interestingly, tyrosine phosphorylation of PLC- γ 1 in this same region (Tyr-771 and Tyr-783) also activates PLC- γ 1 (Nishibe et al., 1990).

A model of PLC- γ 1 folding is shown in Figure 6(b). The model proposes that the X and Y domains physically associate in a manner that protects this region from proteolysis and provides a catalytic site. The results also suggest that sites near the SH3 region of PLC- γ 1 are the most susceptible to V8 cleavage. It seems reasonable that the SH3 domain of PLC- γ 1 is in an exposed position, as SH3 domains are thought to mediate protein-protein interactions. The isolated SH3 domain of PLC- γ 1 has been shown to interact with unidentified proteins in the cytoskeleton (Bar-Sagi et al., 1993) and with dynamin, a GTP-binding protein associated with microtubules (Gout et al., 1993). However, these results have not been demonstrated for the intact PLC- γ 1 molecule.

The authors appreciate critical reading of the manuscript by Dr. Concepcio Soler, technical assistance by Laura Hein and Tatiana Sorkina, and the gift of antibodies by Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD, U.S.A.). Support from grants CA43720 and CA09582 from the National Cancer Institute is acknowledged.

REFERENCES

- Anderson, K., Koch, C. A., Grey, L., Ellis, C., Moran, M. F. and Pawson, T. (1990) *Science* **250**, 979–981
- Arteaga, C. L., Johnson, M. D., Todderud, G., Coffey, R. J., Carpenter, G. and Page, D. L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10435–10439
- Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V. and Schlessinger, J. (1993) *Cell* **74**, 83–91
- Blank, J. L., Foster, K. A. and Hawthorne, J. N. (1991) *J. Neurochem.* **57**, 15–21
- Bristol, A., Ha, S. M., Kriz, R. W., Stahl, M. L., Fan, Y. S., Byer, M. G., Eddy, R. L., Shows, T. B. and Knopf, J. L. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53**, 915–920
- Cifuentes, M., Honkanen, L. and Rebecchi, M. J. (1993) *J. Biol. Chem.* **268**, 11586–11593
- Ellis, V. M., Carne, A. and Katan, M. (1993) *Eur. J. Biochem.* **213**, 339–347
- Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., Nakanishi, O., Suzuki, K. and Takenawa, T. (1989) *J. Biol. Chem.* **264**, 21885–21890
- Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D. and Waterfield, M. D. (1993) *Cell* **75**, 25–36
- Homma, Y. and Takenawa, T. (1992) *J. Biol. Chem.* **267**, 21844–21849
- Jones, G. and Carpenter, G. (1993) *J. Biol. Chem.* **268**, 20845–20850
- Kim, J. W., Sim, S. S., Kim, U. H., Nishibe, S., Wahl, M. I., Carpenter, G. and Rhee, S. G. (1990) *J. Biol. Chem.* **265**, 3940–3943
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Margolis, B., Bellot, F., Honegger, A. M., Ullrich, A., Schlessinger, J. and Zilberstein, A. (1990a) *Mol. Cell. Biol.* **10**, 435–441
- Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T. and Schlessinger, J. (1990b) *EMBO J.* **9**, 4375–4380
- Mohammadi, M., Honegger, A. M., Rotin, D., Fishcher, R., Bellot, F., Li, W., Dionne, C. A., Jaye, M., Rubinstein, M. and Schlessinger, J. (1991) *Mol. Cell. Biol.* **11**, 5068–5078
- Mohammadi, M., Dionne, C. A., Li, W., Li, N., Spivak, T., Honegger, A. M., Haye, M. and Schlessinger, J. (1992) *Nature (London)* **358**, 681–684
- Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G. and Carpenter, G. (1990) *Science* **250**, 1253–1256
- Park, D. and Rhee, S. G. (1992) *Mol. Cell. Biol.* **12**, 5816–5823

- Peters, K. G., Marie, J., Wilson, E., Ives, H. E., Escobedo, J., Del Rosario, M., Mirda, K. and Williams, L. T. (1992) *Nature (London)* **358**, 678–684
- Rhee, S. G., Suh, P. G., Ryu, S. H. and Lee, S. Y. (1989) *Science* **244**, 546–550
- Ronnstrand, L., Mori, S., Arridsson, A. K., Eriksson, A., Wernstedt, C., Hellman, U., Welsh, L. C. and Heldin, C. H. (1992) *EMBO J.* **11**, 3911–3919
- Rotin, D., Margolis, B., Mohammadi, M., Daly, R. J., Daum, G., Li, N., Fischer, E. H., Burgess, W. H., Ullrich, A. and Schlessinger, J. (1992) *EMBO J.* **11**, 559–567
- Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G. and Rhee, S. G. (1987) *J. Biol. Chem.* **262**, 12511–12518
- Savage, C. R. and Cohen, S. (1972) *J. Biol. Chem.* **247**, 7609–7611
- Smercka, A. V., Hepler, J. R., Brown, K. O. and Sternweis, P. C. (1991) *Science* **251**, 804–807
- Soler, C., Beguinot, L., Sorkin, A. and Carpenter, G. (1993) *J. Biol. Chem.* **268**, 22010–22019
- Stahl, M. L., Ferez, C. F., Kelleher, K. L., Kriz, R. W. and Knopf, J. L. (1988) *Nature (London)* **332**, 269–272
- Suh, P. G., Ryu, L. H., Moon, K. H., Suh, H. W. and Rhee, S. G. (1988) *Cell* **54**, 161–169
- Taylor, S. J., Chae, H. Z., Rhee, S. G. and Exton, J. H. (1991) *Nature (London)* **350**, 516–518
- Valius, M., Bazenet, C. and Kazlauskas, A. (1993) *Mol. Cell. Biol.* **13**, 133–143
- Wahl, M. I., Nishibe, S., Kim, J. W., Kim, H., Rhee, S. G. and Carpenter, G. (1990) *J. Biol. Chem.* **265**, 3944–3948
- Wahl, M. I., Jones, G. A., Nishibe, S., Rhee, S. G. and Carpenter, G. (1992) *J. Biol. Chem.* **267**, 10447–10456
- Waldo, G. L., Boyer, J. L., Morris, A. J. and Harden, T. K. (1991) *J. Biol. Chem.* **266**, 14217–14225
- Wu, K., Jiang, H., Katz, A. and Simon, M. (1993) *J. Biol. Chem.* **268**, 3704–3709