Activation of a phospholipase Cβ2 deletion mutant by limited proteolysis

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All phosphoinositide-specific phospholipases C (PLC) identified until today exhibit a high degree of similarity within two regions of 170 and 260 residues, respectively, which are designated regions X and Y. The PLCβ family, including four members designated PLCβ1, PLCβ2, PLCβ3 and PLCβ4, is regulated by heterotrimeric G proteins. In order to investigate structure–function relationships of PLCβ2, we expressed PLCβ2Δ, a deletion mutant of PLCβ2 which lacks most of the sequence downstream of region Y, in the baculovirus/insect cell system. The mutant was present in both soluble and particulate fractions of Sf9 cells and was demonstrated to be catalytically active and sensitive to βγ-subunits. Sf9 cytosol containing this mutant was subjected to limited proteolysis by trypsin and *S. aureus* protease V8, respectively. Immunochemical analysis revealed that both proteases cleaved the enzyme between the regions X and Y. Most interestingly, proteolytic cleavage at this site by both proteases stimulated the catalytic activity of PLC2β2Δ. The proteolytically activated enzyme was still sensitive to βγ-subunits and showed an unchanged concentration dependence on Ca^{2+}. Gel filtration chromatography indicated that the fragments generated by cleavage between the regions X and Y were still connected and formed a functional heterodimeric complex. In order to visualize all fragments generated by protease V8, PLCβ2Δ was purified to homogeneity from Sf9 cytosol. Limited proteolysis of the purified enzyme by *S. aureus* protease V8 and subsequent SDS/PAGE and silver staining revealed that several cuts take place between the regions X and Y and that the resulting fragments remain intact. We hypothesize that the activating proteolytic cut induces a conformational change of the enzyme which might facilitate hydrolysis of the phospholipid substrate.

INTRODUCTION

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns-P_2) to inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol by phospholipase C (PLC) is a key mechanism by which many extracellular signalling molecules like hormones, neurotransmitters and growth factors regulate important functions of their target cells [1,2]. There is increasing diversity among PLC enzymes [3,4]. Until today, the cDNAs of at least ten different PLC isozymes have been cloned from mammalian cells. All PLC isozymes known until today share a high sequence similarity within two regions including about 170 and 250 residues, respectively. These regions are referred to as X and Y and, as suggested by the crystallization studies [5,6] they make up the catalytic core of the enzymes. According to their sequence similarities outside these regions, the PLC isozymes can be divided into three groups designated PLCβ, PLCγ and PLCδ.

The PLCβ group consists of four members designated PLCβ1, PLCβ2, PLCβ3 and PLCβ4. While PLCβ4 is stimulated by G protein α-subunits of the Gq family [7,8], PLCβ1, PLCβ2, and PLCβ3 are sensitive to stimulation by α- as well as βγ-subunits [4]. With respect to activation by βγ-dimers, PLCβ2 [9] and PLCβ3 [10] are major targets. The order of βγ sensitivity is PLCβ3 $\geqslant$ PLCβ2 $\gg$ PLCβ1 [11].

By limited proteolysis [12] and by site-directed mutagenesis [13], αq-subunits have been shown to stimulate PLCβ1 by interaction with a region in the carboxyl terminal portion of the enzyme. In contrast, site-directed mutagenesis of PLCβ2 revealed that the carboxyl terminus is not required for βγ stimulation of PLCβ2 [14–16]. Thus, the interaction of PLCβ enzymes with βγ-subunits appears to take place at a site distinct from the site involved in αq stimulation. However, further investigation of structural requirements for βγ stimulation of PLCβ2 by site-directed mutagenesis of its cDNA was compromised by amino terminal deletion mutants not being expressed in COS-1 cells [15]. Random mutagenesis studies [17] suggested the amino-terminal portion of PLCβ2 containing a putative pleckstrin homology domain being essential for activation of the enzyme by βγ-subunits.

Therefore, in this paper we applied a different approach to obtain more information about structure–function relationships of PLCβ2. We expressed a PLCβ2 mutant we had already extensively characterized in COS-1 cells in the baculovirus/insect cell system in order to obtain large amounts of recombinant PLC. This PLC was then subjected to limited proteolysis by two proteases, trypsin and *S. aureus* protease V8. The construct we chose for this study was PLCβ2Δ[F819-E1166], the smallest PLCβ mutant which had been functional in COS-1 cells. This construct contained a large deletion between the Y region and the carboxyl terminus of PLCβ2. Its catalytic activity and Ca^{2+} dependence were similar to the wild-type enzyme. The deletion mutant was still fully sensitive to stimulation by βγ-subunits. Compared with the wild-type enzyme, a higher proportion of PLCβ2Δ[F819-E1166] was present in the soluble fraction of COS-1 cells. The effects of limited proteolysis of this well-characterized PLC mutant on its catalytic activity and its regulation by G protein βγ-subunits are presented in this paper.

EXPERIMENTAL

Plasmid construction

The cDNA of PLCβ2Δ[F819-E1166] was obtained by site-directed mutagenesis as described [15]. The 5′-noncoding region was removed by PCR amplification of the coding region using

Abbreviations used: G protein, guanine nucleotide-binding protein; InsP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PLCβ2Δ, deletion mutant PLCβ2Δ[F819-E1166]; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; Sf9 cells, insect cells from *Spodoptera frugiperda*; TIM barrel, triosephosphate isomerase-like barrel structure.

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the oligonucleotide primers 5′-TTTGAATTCCACCATGGCT-CTGCTCAACCCTGTC-3′ (upstream, sense) and 5′-TTTGA-ATTCAGAGGCGGCTCTCCTGGGC-3′ (downstream, antisense). The upstream primer introduced an *Eco*RI and a *Nco*I restriction site into the cDNA. The latter modification caused a serine to alanine replacement in position 2 of the mutant polypeptide, PLCβ2Δ[F819-E1166]S2A. This protein is referred to as PLCβ2Δ throughout this paper. The PCR product was subcloned into pBluescript II SK (Stratagene) and the *Eco*47III–*Not*I fragment was replaced by the corresponding fragment of the PLCβ2[F819-E1166] DNA which had been sequenced before [15]. The remaining PCR-amplified region was verified by DNA sequencing. The resulting PLCβ2Δ cDNA was subcloned into the *Eco*RI site of the baculovirus transfer vector pVL1393 (Invitrogen).

Cell culture and production of recombinant baculovirus

Spodoptera frugiperda (Sf9) cells were cultured at 27 °C in TNM-FH medium (Sigma) supplemented with 10 % fetal calf serum, 50 μg/ml gentamycin (Sigma) and 2.5 μg/ml amphotericin B (Gibco). Recombinant baculoviruses were obtained as described [18] by transfection of Sf9 cells with a 10:1 mixture of plasmid and modified baculovirus DNA (Baculogold, Pharmingen). This DNA contains a lethal deletion and the virus is rescued by recombination with the plasmid DNA. The recombinant baculovirus was amplified through two cycles of infection of 6×10^6 Sf9 cells, resulting in a virus titre of approx. 2×10^8/ml.

Expression of PLCβ2Δ

Several 55 cm^2 dishes containing 6×10^6 Sf9 cells each were incubated with recombinant baculovirus at a multiplicity of infection (MOI) of 5 in a volume of 2 ml for 1 h at 27 °C. Subsequently, cells were maintained as described above for the times indicated in the figure legends. Cells were then rinsed twice with phosphate-buffered saline (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 pH 7.2, 2.6 mM KCl, 136 mM NaCl) and scraped into 1 ml per dish of hypotonic buffer (20 mM Tris/Cl pH 7.5, 2 mM EDTA, 2 μg/ml soybean trypsin inhibitor, 3 mM benzamidine, 1 μM pepstatin, 1 μM leupeptin, 100 μM phenylmethylsulphonyl fluoride). After two freeze/thaw cycles the homogenate was centrifuged in a Beckman JA-20 rotor at 2500 ***g*** for 1 min at 4 °C to remove unbroken cells and nuclei. The supernatant was recentrifuged in the same rotor at 48400 ***g*** for 20 min at 4 °C. The supernatant containing the soluble proteins was transferred to a fresh tube and the pellet containing the particulate fraction was washed once in hypotonic buffer. The preparations were snap-frozen in liquid nitrogen and stored at −80 °C. For the assessment of the time-course of expression, whole-cell detergent extracts were prepared as follows. Cells were scraped into 500 μl per dish extraction buffer (20 mM Tris/HCl pH 7.5, 5 mM EDTA, 10 mM EGTA, 1.6 % (w/v) sodium cholate, 3 mM benzamidine, 0.3 % (v/v) 2-mercaptoethanol, 100 μM phenylmethylsulphonyl fluoride), kept on ice for 1 h and vortexed briefly every 10 min. The homogenate was centrifuged in a Beckman JA-20 rotor at 27700 ***g*** for 20 min at 4 °C. The supernatant was snap-frozen and stored at −80 °C. Expression of the ≈100 kDa PLCβ2Δ was first detected 2 days after infection, reached its maximum after 3 days, decreased rapidly after 4 days and was not detectable anymore after 5 days. PLCβ2Δ was present and catalytically active in both particulate and soluble fractions prepared from Sf9 cells infected with the recombinant virus.

Partial purification of PLCβ2Δ by chromatography on Mono-Q

Cytosol (10 mg protein) of Sf9 cells expressing PLCβ2Δ was applied to a Mono-Q HR 5/5 column (Pharmacia, bed volume 1 ml) which had been equilibrated with buffer A (20 mM Tris/HCl pH 7.0, 1 mM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulphonyl fluoride). The flow rate was 1 ml/min. After application of the sample, the column was washed with 4 ml of buffer A and eluted at the same flow rate with a linear gradient (5 ml) of NaCl (0–1 M) in buffer A followed by 5 ml of buffer B (= buffer A containing 1 M NaCl). Fractions of 0.5 ml were collected and analysed by Western blotting and determination of PLC activity. PLCβ2Δ eluted from this resin at approximately 360 mM NaCl. Cytosol (10 mg protein) from Sf9 cells expressing β-galactosidase was chromatographed in the same way to generate a control preparation devoid of recombinant PLCβ2Δ.

Limited proteolysis of PLCβ2Δ with trypsin

Twenty-five μl of the peak fraction from the Mono-Q chromatography (200 μg protein) was incubated with increasing amounts of TPCK-treated trypsin (Sigma) in 45 μl of 50 mM Tris/HCl pH 7.0 for 15 min at 37 °C. The reaction was terminated by addition (30 μl) of soybean trypsin inhibitor (Sigma) and aprotinin (Bayer) to final concentrations of 10 and 100 μg/ml, respectively.

Limited proteolysis of PLCβ2Δ with protease V8

Three hundred μg (21 μl) of cytosol from Sf9 cells expressing PLCβ2Δ were incubated with increasing amounts of *S. aureus* protease V8 (Boehringer) in 100 μl of 50 mM sodium phosphate pH 7.8 for 15 min at 37 °C. The reaction was terminated by addition of 100 μg α2-macroglobulin (Boehringer) in 50 μl of 50 mM sodium phosphate pH 7.8.

Gel filtration of *S. aureus* protease V8-treated PLCβ2Δ on Superdex-75

Cytosol from Sf9 cells expressing PLCβ2Δ was subjected to limited proteolysis by *S. aureus* protease V8 as described above. Subsequently, the sample (2 mg protein in a volume of 250 μl) was applied to a Superdex-75 HR 10/30 column (Pharmacia, bed volume 30 ml) equilibrated and run at a flow rate of 0.5 ml/min buffer (20 mM Tris/HCl pH 7.0, 0.5 mM DTT, 500 mM NaCl, 1 % (w/v) sodium cholate). The initial 5 ml of buffer was discarded, then 65 fractions of 250 μl were collected.

PLC assay

Inositol phosphate formation was assayed for 30 min at 25 °C as previously described [9,15] using exogenous phospholipid vesicles containing [^{3}H]PtdInsP_2 as substrate. The reaction mixture (70 μl) contained 280 μM phosphatidylethanolamine, 28 μM [^{3}H]PtdInsP_2 (5 Ci/mol), 50 mM Tris/maleate, pH 7.0, 10 mM LiCl, 10 mM 2,3-diphosphoglycerate, 3 mM EGTA, and free Ca^{2+} and sodium deoxycholate at concentrations given in the figure legends. The reaction was terminated by adding 350 μl of chloroform/methanol/concentrated HCl (500:500:3, by volume) and vortexing. Samples were then supplemented with 100 μl of 1 M HCl containing 5 mM EGTA. Phase separation was accelerated by centrifugation for 1 min in an Eppendorf microcentrifuge. A 200 μl portion of the aqueous phase was

Table 1 Purification of PLCβ2Δ

The purification of PLCβ2Δ is outlined in Experimental.

Step	Vol. (ml)	Protein (mg)	Specific activity (μmol · min^{-1} · mg^{-1})	Purification (-fold)	Recovery (%)
Sf9 Cytosol	4	8	0.3	1	100
Mono-Q	2.5	0.75	1.3	4.3	42
Heparin Sepharose	2.5	0.16	4.9	16.3	33
Hydroxyapatite	1.5	0.009	16.2	53.9	6

supplemented with scintillation fluid and radioactivity was measured in a scintillation counter.

Purification of PLCβ2Δ from Sf9 cytosol

The purification of PLCβ2Δ is summarized in Table 1 and illustrated by Figure 2. Cytosol from Sf9 cells (8 mg protein) expressing PLCβ2Δ was applied to a Mono-Q anion exchange column as described above. Fractions containing PLCβ2Δ were pooled (0.75 mg protein), concentrated approximately 10-fold using Centricon-10 microconcentrators (Amicon), diluted 6-fold with buffer A and then applied to a column (600 μl bed volume) of Heparin Sepharose CL-6B (Pharmacia) equilibrated with buffer A. The flow rate was 0.5 ml/min. After washing the column with 5 ml buffer A, the sample was eluted with a linear gradient (10 ml) of NaCl (0–1 M) in buffer A followed by 10 ml of buffer B (= buffer A containing 1 M NaCl) at the same flow rate. Fractions of 500 μl were collected and the peak fractions were pooled (160 μg protein), diluted with an equal volume of buffer C (20 mM Tris/HCl pH 7.5, 100 μM EDTA, 1 mM dithiothreitol, 100 μM phenylmethylsulphonyl fluoride) and applied to a hydroxyapatite column (Calbiochem, HPLC grade, bed volume 400 μl) equilibrated with buffer C. The flow rate was 0.5 ml/min. The resin was washed with 3 ml of buffer C and eluted with a linear gradient (5 ml) of potassium phosphate pH 7.5 (0–500 mM) in buffer C followed by 5 ml of buffer C containing 500 mM potassium phosphate pH 7.5. Fractions of 500 μl were collected. A single peak containing approx. 9 μg of mostly homogeneous PLCβ2Δ was obtained at about 200 mM potassium phosphate. This corresponded to a double band of $\approx$ 100 kDa on a silver gel (Figure 2) without further contaminants. Copurification of the two proteins with a similar molecular weight raised the possibility that the lower band represented a proteolytic fragment of the full-length PLCβ2Δ. If so, the enzyme would be truncated at the carboxyl terminus as the carboxyl terminal antibody recognized only one polypeptide (not shown). The pure enzyme exhibited a specific activity of $\approx$ 16.2 μmol · min^{-1} · mg^{-1} protein as assessed in the presence of 1 mM Ca^{2+} and 3.4 mM sodium deoxycholate. This is similar to the specific activity measured for native PLCβ purified from bovine brain (12.9 μmol · min^{-1} · mg^{-1} protein) [19].

Miscellaneous

The purification of $\beta\gamma_t$ from bovine rod outer segments has been described previously [9]. Antibodies to the carboxyl-terminal pentadecapeptide of human PLCβ2 were prepared as described elsewhere [20]. Antibodies raised against the peptide LSRIYPKG within the Y region of PLCβ1 were obtained from Dr. Sue Goo Rhee, Bethesda, MD, U.S.A. SDS/PAGE and immunoblotting were performed as described [9,15]. Protein concentrations were determined according to Bradford [21] using bovine IgG as standard.

RESULTS AND DISCUSSION

PLCβ2Δ was used to investigate structure–function relationships of PLCβ2. To this end, limited proteolysis of a partially purified cytosol preparation was performed with increasing concentrations of TPCK-treated trypsin. Figure 3 shows immunoblots of a typical trypsinization experiment. In Figure 3A, antibodies raised against the carboxyl terminal 15 residues of PLCβ2 were used. These antibodies recognized a tryptic fragment of $\approx$ 70 kDa present in lanes 2–4 which was generated by tryptic cleavage upstream of the X region (cf. Figure 1). At higher trypsin

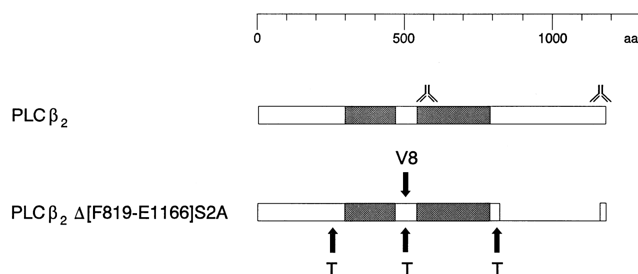

Figure 1 Linear representation of PLCβ2 wild-type and PLCβ2Δ

The two shaded regions represent the regions X and Y sharing high similarity in all known phosphoinositide-specific phospholipase C isozymes. The amino acid numbering (aa) is indicated. The cDNA of PLCβ2Δ was constructed as described in Experimental. The arrows at the bottom indicate the hypothetical cleavage sites by trypsin (T), the arrows at the top the cleavage sites of *S. aureus* protease V8 (V8). The symbols above the representation of PLCβ2 indicate the recognition sites of the antibodies used.

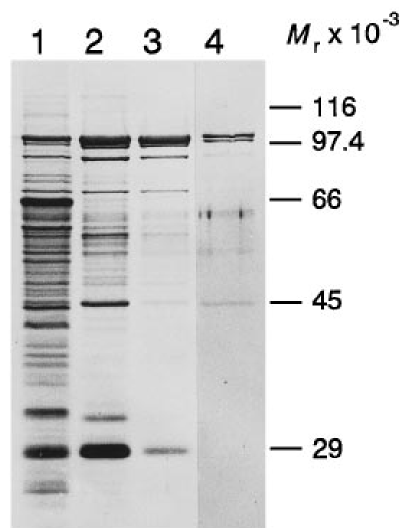

Figure 2 Purification of PLCβ2Δ

Sf9 cells were infected for 4 days with baculovirus encoding PLCβ2Δ. The protein was purified from the soluble fraction by sequential chromatography on Mono-Q, Heparin Sepharose and hydroxyapatite as described in Experimental. Aliquots of the crude cytosol (20 μg protein; lane 1), and the peaks obtained by chromatography on Mono-Q (20 μg protein; lane 2), Heparin Sepharose (4 μg protein; lane 3) and hydroxyapatite (50 μl of peak fraction; lane 4) were subjected to SDS/PAGE and stained with silver. The positions of the molecular mass standards are indicated.

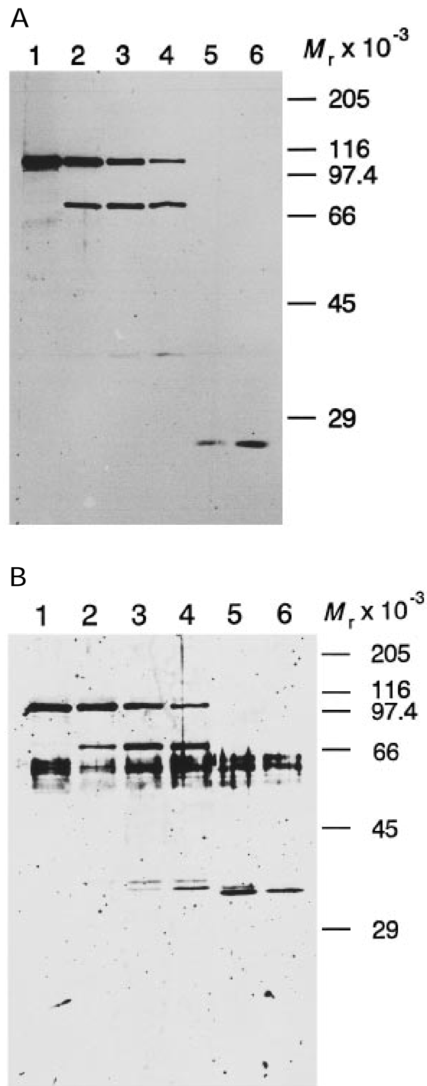

Figure 3 Limited proteolysis of partially purified PLCβ2Δ by trypsin

PLCβ2Δ was partially purified from the soluble fraction of baculovirus-infected Sf9 cells by Mono-Q anion exchange chromatography as described in Experimental. Aliquots (200 μg protein) of the peak fraction were incubated in the absence of trypsin (lane 1), or in the presence of 50 ng (lane 2), 100 ng (lane 3), 200 ng (lane 4), 500 ng (lane 5), or 1000 ng (lane 6) trypsin for 15 min at 37 °C as described in Experimental. One fourth of the reaction volume was subjected to SDS/PAGE and immunoblotting was performed using antibodies reactive against the carboxyl terminus of PLCβ2Δ (**A**) or a peptide sequence within the Y region (**B**). The positions of the molecular mass standards are indicated. The staining between the 66 and 45 kDa marker proteins was also evident in lanes containing no sample (not shown) and therefore considered unspecific.

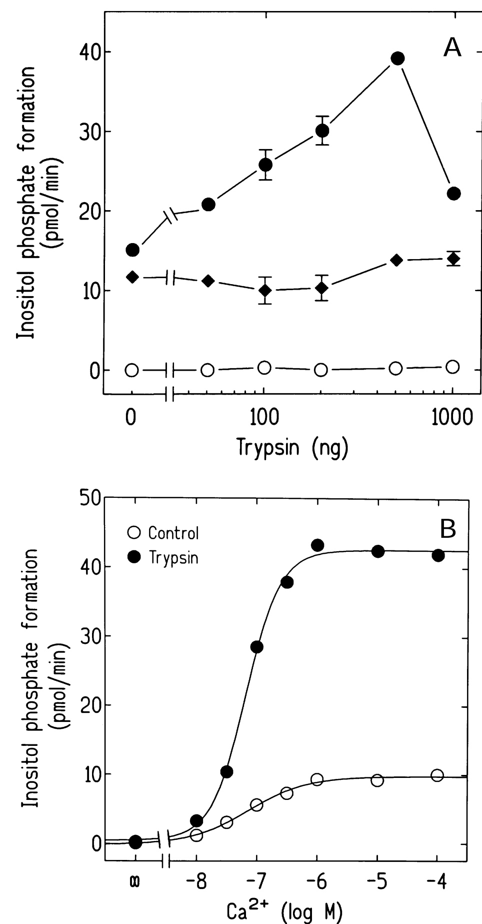

Figure 4 Effect of trypsin on inositol phosphate formation by partially purified PLCβ2Δ

Soluble fractions from Sf9 cells expressing PLCβ2Δ or β-galactosidase, respectively, were subjected to anion exchange chromatography on Mono-Q to partially purify PLCβ2Δ and to obtain a control preparation, respectively. (**A**) Aliquots of the fraction containing the peak of PLCβ2Δ (closed circles) activity or of the corresponding control fraction (open circles) were treated with the indicated amounts of trypsin and assayed for inositol phosphate formation in the presence of 1 mM free Ca^{2+} and 3.4 mM sodium deoxycholate. As a control (closed diamonds), the non-trypsinized peak fraction containing PLCβ2Δ (closed circles) was assayed in the presence of the control which had been treated with the indicated amounts of trypsin. The values correspond to the means $\pm$ S.D. of triplicate determinations. (**B**) Aliquots of the fraction containing the peak of PLCβ2Δ activity were incubated in the absence (open circles) or presence (closed circles) of 500 ng trypsin and assayed for inositol phosphate formation at 3.4 mM sodium deoxycholate and the concentrations of free Ca^{2+} indicated at the abscissa. The values correspond to the means of duplicate determinations.

concentrations, this N-terminally truncated fragment was not detected anymore. Control experiments showed that the $\approx$ 20 kDa protein present in lanes 5 and 6 was not a proteolytic fragment, but trypsin itself. The immunoblot shown in Figure 3B was performed with antibodies raised against the peptide LSRIYPKG within the Y region of PLCβ1. The corresponding sequence of PLCβ2 differs only in one amino acid and the antiserum has previously been shown to recognize both PLCβ1 and PLCβ2 [22]. The anti-peptide antibody to the Y region detected three fragments between $\approx$ 33 and $\approx$ 35 kDa which were not recognized by the carboxyl terminal antibody. Therefore, at least one cut must take place between the antibody recognition site and the carboxyl terminus of the enzyme. The upstream cleavage site(s) yielding the $\approx$ 33–35 kDa fragments could be located between the X and the Y regions (cf. Figure 1) as calculated from the molecular masses of the three fragments. This hypothesis is supported by the finding that the $\approx$ 33–35 kDa fragments were not recognized by anti-peptide antibodies to the X region of PLCβ (not shown). The effect of limited proteolysis of PLCβ2Δ by trypsin on the catalytic function of the enzyme was also studied. Inositol phosphate formation by the same trypsin-treated samples were as immunoblotted in Figure 3 was measured in the presence of 1 mM free Ca^{2+} and 3.4 mM sodium deoxycholate (Figure 4A). Most interestingly, the catalytic activity of the recombinant PLC increased with increasing trypsin

Table 2 Effect of trypsin on sensitivity of partially purified PLCβ2Δ to stimulation by G protein $\beta\gamma$-subunits

	Control	$\beta\gamma_t$
Control	0.5 ± 0.2	31.9 ± 1.2
Trypsin	15.2 ± 0.4	32.2 ± 1.8

concentrations (closed circles), although the amount of immuno-detectable protein clearly decreased (cf. Figures 3A and 3B). Maximal PLC activity was observed at 500 ng trypsin corresponding to lane 5 in Figure 3. At 1000 ng trypsin, the catalytic activity was lower than at 500 ng. This decrease is probably due to further proteolytic degradation of the active fragments. In order to confirm that it was the recombinant and not the endogenous PLC from Sf9 cells that was activated by trypsin, cytosol of Sf9 cells expressing β-galactosidase which had been chromatographed and trypsinized exactly like PLCβ2Δ-containing cytosol was assayed for PLC activity (open circles). This control sample exhibited no detectable activity even at the high Ca^{2+} (1 mM) and deoxycholate (3.4 mM) concentrations present in this assay and no activation by trypsin was observed. In order to exclude the possibility that some protein other than PLC present in Sf9 cytosol was proteolysed and thus became capable of activating the recombinant PLC, an additional control was performed (closed diamonds): The non-trypsinized cytosol preparation containing PLCβ2Δ was reconstituted with the trypsin-treated cytosol preparation of β-galactosidase-expressing cells. No activation of PLC activity was observed under these conditions. Thus we conclude that PLCβ2Δ was activated by proteolytic cleavage with trypsin. As the $\approx$ 70 kDa proteolytic fragment was not present at 500 ng trypsin (cf. lane 5 in Figures 3A and 3B), the concentration that resulted in the maximal PLC activation (Figure 4A), it appears likely that the increase of catalytic activity was due to the generation of one of the $\approx$ 33–35 kDa fragments, one of which showed a peak of immunoreactivity in lane 5 (Figure 3B). Thus we hypothesize that the PLC-activating cut by trypsin might take place between the regions X and Y. This intervening sequence contains three potential trypsin cleavage sites. We next investigated whether the proteolytically activated PLCβ2Δ was still sensitive to stimulation by G protein $\beta\gamma$-subunits. Non-trypsinized PLCβ2Δ (cf. Figure 3, lane 1) and PLCβ2Δ treated with 500 ng trypsin (cf. Figure 3, lane 5) were assayed for PLC activity in the absence or presence of 1.7 μM $\beta\gamma_t$ (Table 2). In the absence of $\beta\gamma_t$, the dramatic stimulation of PLC activity by trypsin became obvious. Moreover, the proteolytically activated enzyme was still further stimulated by exogenous $\beta\gamma$-subunits. Interestingly, the $\beta\gamma$-stimulated PLC activity was similar in both trypsinized and non-trypsinized PLCβ2Δ. The dose-dependence of both non-trypsinized and trypsinized PLCβ2Δ on free Ca^{2+} was assayed in the presence of 3.4 mM sodium deoxycholate (Figure 4B). The maximal PLC activity of both samples was reached at 1 μM free Ca^{2+}. Trypsin treatment increased the maximal PLC activity about 4-fold, while the EC_{50} values for half-maximal stimulation by Ca^{2+} were very similar.

In order to test the hypothesis that PLCβ2Δ is activated by proteolytic cleavage between the X and the Y regions, we subjected the enzyme to limited proteolysis by a second protease. As the stretch of sequence between the regions X and Y is particularly rich in Glu residues, we used *S. aureus* protease V8 (= endoproteinase Glu-C) for our further studies. In the experiment shown in Figure 5, cytosol from Sf9 cells expressing PLCβ2Δ (300 μg protein) was incubated with increasing amounts of *S. aureus* protease V8, subjected to SDS/PAGE and immunoblotted with antibodies raised against the carboxyl terminus of PLCβ2. With increasing concentrations of protease V8, the amount of $\approx$ 100 kDa PLCβ2Δ gradually decreased. In contrast, four proteolytic fragments of $\approx$ 33–38 kDa were detected in lanes 2–4, corresponding to 0.6–3.0 μg *S. aureus* protease V8. The largest of these four fragments was further proteolysed and the resulting three fragments (lane 5) proved to be stable even at high concentrations of protease V8. As all fragments detected by the antibody used contain the carboxyl terminus, it is possible to calculate where the proteolytic cuts take place. All four cleavage sites yielding the fragments detected in Figure 5 are located between the regions X and Y (cf. Figure 1). If the above hypothesis that proteolytic cleavage between the regions X and Y activates the enzyme is true, limited proteolysis by *S. aureus* protease V8 should, just like trypsin treatment, stimulate the activity of PLCβ2Δ. The experiment shown in Figure 6, which was performed with the same samples as the immunoblot (Figure 5), demonstrates that this is the case. The enzyme activity of PLCβ2Δ was increased approximately 2-fold by treatment with *S. aureus* protease V8. In contrast, treatment of cytosol from Sf9 cells expressing β-galactosidase did not result in increased PLC activity (not shown). Neither did the reconstitution of non-proteolysed PLCβ2Δ with proteolysed cytosol containing β-galactosidase lead to a stimulation of PLC activity (not shown). These results indicate that, similar to trypsin, *S. aureus* protease V8 activates PLCβ2Δ by proteolytic cleavage between the regions X and Y. The V8-activated enzyme was also investigated for its sensitivity to G protein $\beta\gamma$-subunits and for its Ca^{2+} dependence. Consistent with the results obtained with trypsin, V8-activated PLCβ2Δ was still sensitive to stimulation by $\beta\gamma$-subunits and showed a dose-dependence on Ca^{2+} that was similar to the non-proteolysed enzyme (not shown).

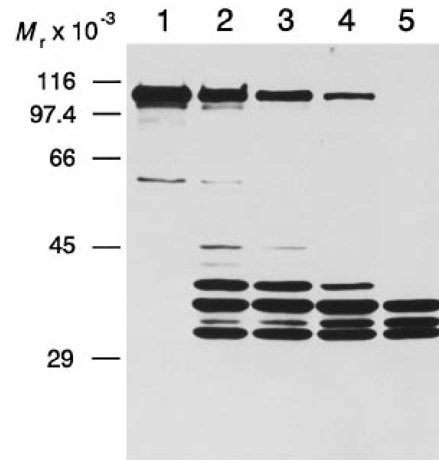

Figure 5 Limited proteolysis of PLCβ2Δ with *S. aureus* protease V8

Cytosol (300 μg protein) from Sf9 cells expressing PLCβ2Δ were incubated in the absence of protease V8 (lane 1), or in the presence of 0.6 μg (lane 2), 1.2 μg (lane 3), 3.0 μg (lane 4), or 6.0 μg (lane 5) protease V8 for 15 min at 37 °C as described in Experimental. Aliquots (50 μg) of the reaction were subjected to SDS/PAGE and immunoblotting was performed using antibodies against the carboxyl terminus of PLCβ2Δ.

Ellis et al. [23] studied structure–function relationships of PLCδ1 by bacterial expression and limited proteolysis with trypsin. These authors observed a moderate activation of PLCδ1

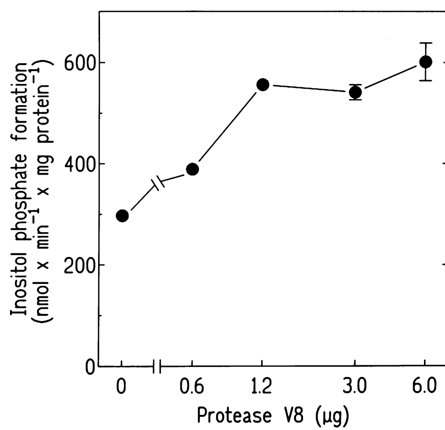

Figure 6 Effect of *S. aureus* protease V8 on inositol phosphate formation by PLCβ2Δ

Cytosol (300 μg) from Sf9 cells expressing PLCβ2Δ was incubated with the indicated amounts of protease V8 and inositol phosphate formation was measured in the presence of 1 mM free Ca^{2+} and 3.4 mM deoxycholate as described in Experimental. The values correspond to the means $\pm$ S.D. of triplicate determinations.

by trypsin. In the maximally activated fractions they detected two major tryptic fragments of approx. 30 and 40 kDa. Amino acid sequencing of these fragments revealed one cleavage site in the amino terminal portion of the molecule and one between the regions X and Y after R474. According to our findings and the similarity of PLCβ and PLCδ enzymes within the regions X and Y, we would predict the latter cleavage site to be responsible for the catalytic activation. However, this issue was not further scrutinized by the authors. Cifuentes et al. [24] studied the effect of trypsin on purified native PLCδ1 and found that this enzyme was cleaved at a site close to the amino terminus yielding a 77 kDa fragment which was further proteolysed at a site between the homologous regions X and Y yielding two fragments of 45 and 32 kDa, respectively. The latter cut was assumed to take place between residues 473 and 491, which is in perfect agreement with the study of Ellis et al. [23]. However, the proteolysis of native PLCδ1 by trypsin was not accompanied by an increase of its catalytic activity. The reason for this discrepancy is unclear, but might lie in the different assay conditions applied.

Next, we investigated the question whether the activating cut between regions X and Y completely separated the two resulting fragments or whether they were still connected by noncovalent interactions. To this end, *S. aureus* protease V8-treated PLCβ2Δ was subjected to a Superdex-75 gel filtration column. An immunoblot of the fractions with the carboxyl terminal antibody is shown in Figure 7A. Most interestingly, the proteolytic fragments which migrate like $\approx$ 35 kDa proteins in SDS gels under denaturing conditions, were eluted before transferrin, the 81 kDa standard protein. The inositol phosphate formation by the column fractions was assayed and is shown in Figure 7B. The peak of PLC activity coincided with the peak of immunoreactivity. These findings provide evidence that the proteolytic fragments generated by the activating cut by protease V8 are still connected and can thus form a heterodimeric functional complex.

The antibody used for the analysis of the fragments generated by *S. aureus* protease V8 only recognized the carboxyl terminal fragment of the heterodimeric complex. In order to identify the amino terminal component of the active complex, PLCβ2Δ was purified from Sf9 cytosol by a three-step chromatographic procedure. The results of the purification are summarized in Table 1 and illustrated in Figure 2. After purification to homogeneity, PLCβ2Δ was subjected to limited proteolysis. The resulting fragments were analysed by SDS/PAGE and silver staining (Figure 8). There was one group of fragments with a molecular weight of $\approx$ 35 kDa corresponding to the fragments recognized by the carboxyl terminal antibody (cf. Figure 6). A second group of fragments was detected at $\approx$ 60 kDa. This finding suggests that the amino-terminal fragment of the *S. aureus* protease V8-proteolysed heterodimeric complex remains intact. Although it is not possible to exactly match one fragment of the $\approx$ 60 kDa group with a corresponding member of the $\approx$ 35 kDa group, it appears likely that one cut between the regions X and Y of the intact enzyme generates two fragments, one of each group. The plurality of fragments in each group is most likely due to the high abundance of Glu residues and thus of potential *S. aureus* protease V8 cleavage sites between the regions X and Y.

Both studies on trypsin digestion of PLCδ1 cited above, either by gel filtration [23] or density gradient centrifugation [24], also provide evidence for the $\approx$ 30 and $\approx$ 40 kDa fragments forming

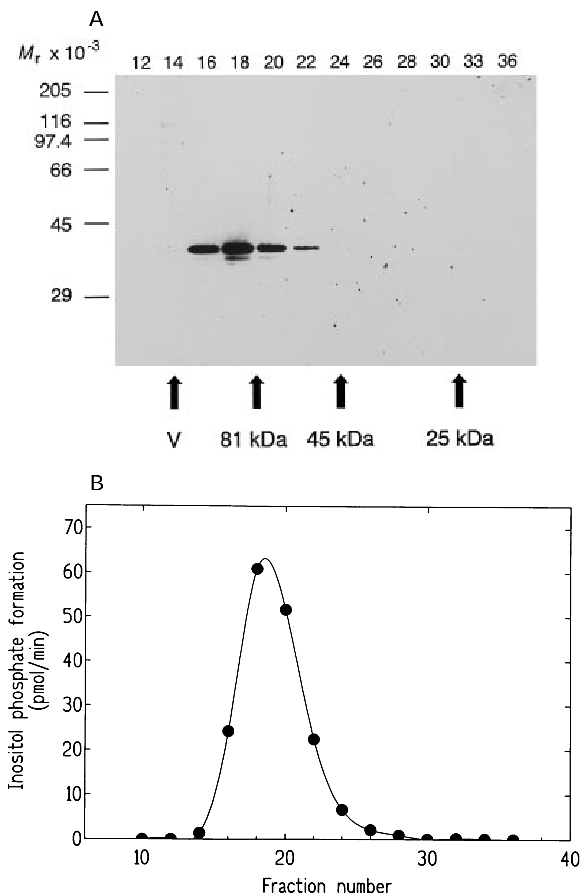

Figure 7 Gel filtration of *S. aureus* protease V8-treated PLCβ2Δ through Superdex-75

Cytosol (1.3 mg protein) of Sf9 cells expressing PLCβ2Δ which had been pre-treated with *S. aureus* protease V8 (5.2 μg) was subjected to a Superdex-75 gel filtration column as described in Experimental. (**A**) Aliquots (50 μl) of the fractions were analysed by SDS/PAGE and immunoblotting using antibodies reactive against the carboxyl terminus of PLCβ2. The positions of the standard proteins (human transferrin, 81 kDa; ovalbumin, 45 kDa; chymotrypsinogen A, 25 kDa) used to calibrate the column and the void volume (V) are indicated at the bottom. (**B**) Aliquots (0.2 μl) of the fractions were assayed for inositol phosphate formation in the presence of 1 mM free Ca^{2+} and 3.4 mM sodium deoxycholate for 30 min at 25 °C. The values correspond to the means of duplicate determinations.

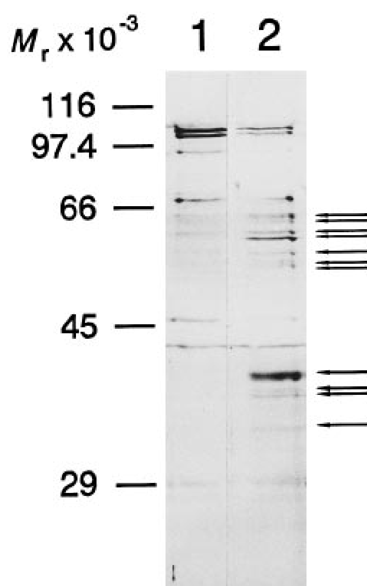

Figure 8 Limited proteolysis of purified PLCβ2Δ with *S. aureus* protease V8

Purified PLCβ2Δ (25 μl of the peak fraction) was incubated in the absence of protease V8 (lane 1) or in the presence of 300 ng protease V8 (lane 2) for 15 min at 37 °C. The samples were subjected to SDS/PAGE and stained with silver. The arrows on the right indicate the proteolytic fragments obtained by treatment with protease *S. aureus* protease V8. The positions of the molecular mass standards are indicated on the left.

one functional heterodimeric complex. This is consistent with our data concerning PLCβ2Δ and suggests that the proteolytic activation which is observed in PLCδ1 as well as PLCβ2 might be due to a conformational change of the enzymes rather than complete removal of a fragment. Homologies with other phospholipid-binding proteins like gelsolin and other actin-binding proteins [25] suggest that the carboxyl terminus of the X region contains a phospholipid-binding site. The finding that a peptide corresponding to this putative phospholipid-binding site stimulates PLCβ2Δ activity [26] further supports this hypothesis. The mechanism of this stimulation could be either a presentation of the substrate by the peptide that facilitates hydrolysis by PLCβ2 or the removal of a hypothetical inhibitory constraint. Site-directed mutagnesis of PLCγ1 [27], PLCγ2 [28] and PLCβ [13] as well as crystallization of PLCδ1 [5] supports the hypothesis that both regions X and Y are essential for catalytic activity. The latter study demonstrated that the regions X and Y form a catalytic TIM barrel (triosephosphate isomerase-like) domain. The stretch of charged residues between X and Y, each of which makes up one half of the TIM barrel architecture, are dispensable for PLCδ1 activity [23]. It is tempting to speculate that the stimulation of catalytic activity might be due to an increased mobility of the X relative to the Y region. However, structural data obtained by crystallization of PLCδ1 [5] do not support this view as the TIM barrel structure composed of the regions X and Y is relatively rigid in the region corresponding to the stretch of sequence between X and Y. However, there is a flexible loop region within the half of the TIM barrel made up by the Y region [5]. Conformational changes affecting this loop might facilitate access of the phospholipid substrate to the catalytic cleft. However, it must be stated that the only crystal structure identified so far is that of PLCδ1 and a corresponding structure of PLCβ isozymes has not yet been proven, although it appears likely. Taken together, the findings on structure–function relationships of PLCγ and PLCδ isozymes parallel our observations regarding structural requirements for catalytic activity of PLCβ2. Thus none of the proteolytic fragments generated by cleavage between the regions X and Y would be predicted to be catalytically active, but the formation of a heterodimeric functional complex would retain PLC activity.

Limited proteolysis of PLCβ1 with calpain [12] removed a large stretch of sequence downstream of region Y, which resulted in a loss of αq sensitivity. The interaction of PLCβ isozymes with βγ-subunits has been demonstrated to take place at a site distinct from the αq interaction site [13–17]. The amino-terminal region of PLCβ2 contains a putative pleckstrin homology domain which is present in many signal-transducing molecules, some of which, e.g. β-adrenergic receptor kinase [29], interact with G protein βγ-subunits. The mutagenesis study of Brazil et al. [17] suggests an interaction of PLCβ2 with βγ-subunits within the pleckstrin homology domain, namely residues Asn86–Asn92. Our present study shows that a deletion mutant of PLCβ2 which lacks most of the sequence downstream of region Y, is still sensitive to βγ-subunits even after limited proteolysis with trypsin or *S. aureus* protease V8. This is not surprising as our data also suggest that the proteolytic fragments produced by cleavage between the regions X and Y are still connected to a functional heterodimeric complex. The sensitivity of the proteolysed enzyme as well as the fragments obtained by *S. aureus* protease V8 proteolysis of purified PLCβ2Δ supports the view that the amino-terminal proteolytic fragment remains intact.

In conclusion, our results indicate that proteolytic cleavage of PLCβ2 between the regions X and Y activates the enzyme. The proteolytic fragments form one functional heterodimeric complex. The exact molecular mechanism, however, will be subject to further experimentation. One useful approach to elucidate this question might be to express fragments upstream and downstream of the putative cleavage sites separately and to investigate the sequence requirements for the formation of a functional complex. If successful, such studies could be an important step towards understanding the catalytic mechanism of PLCβ enzymes.

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