Regulation of Apoptosis by Phosphatidylinositol 4, 5 Bisphosphate Inhibition of Caspases, and Caspase Inactivation of Phosphatidylinositol Phosphate 5 Kinases

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Abbreviations: PIP2, phosphatidylinositol 4,5 bisphosphate; PIP3, phosphatidylinositol 3,4,5 triphosphate, PIP5K1, phosphatidylinositol phosphosphate 5 kinase type I
Summary

Phosphoinositides such as PI(3,4,5)P$_3$ and PI(3,4)P$_2$ promote cell survival and protect against apoptosis by activating Akt/PKB which phosphorylates components of the apoptotic machinery. We now report that another phosphoinositide, PI(4,5)P$_2$ [PIP$_2$] is a direct inhibitor of initiator caspases-8 and -9, and their common effector caspase-3. PIP$_2$ inhibited procaspase-9 processing in cell extracts and in a reconstituted procaspase-9/Apaf1 apoptosome system. It inhibited purified caspase-3 and -8 activity, at physiologically attainable PIP$_2$ levels in mixed lipid vesicles. Caspase-3 binding to PIP$_2$ was confirmed by cosedimentation with mixed lipid vesicles. Overexpression of phosphatidylinositol phosphate 5-kinase $\alpha$ (PIP5K$\alpha$), which synthesizes PIP$_2$, suppressed apoptosis, while a kinase-deficient mutant did not. Protection by the wildtype PIP5K$\alpha$ was accompanied by decreases in the generation of activated caspases and of caspase-3 cleaved PARP. Protection was not mediated through PIP$_3$ or Akt activation. An anti-apoptotic role for PIP$_2$ is further substantiated by our finding that PIP5K$\alpha$ was cleaved by caspase-3 during apoptosis, and cleavage inactivated PIP5K$\alpha$ in vitro. Mutation of the P$_4$ position (D279A) of the PIP5K$\alpha$ caspase-3 cleavage consensus prevented cleavage in vitro, and during apoptosis in vivo. Significantly, the caspase-3 resistant PIP5K$\alpha$ mutant was more effective in suppressing apoptosis than the wildtype kinase. These results show that PIP$_2$ is a direct regulator of apical and effector caspases in the death-receptor and mitochondrial pathways, and that PIP5K$\alpha$ inactivation contributes to the progression of apoptosis. This novel feedforward amplification mechanism for maintaining the balance between life and death of a cell works through phosphoinositide regulation of caspases and caspase regulation of phosphoinositide synthesis.
Introduction

Phosphoinositides have major roles in intracellular signaling and cell proliferation. The D3 phosphorylated phosphoinositides, phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$) and PI(3,4)P$_2$, have been clearly implicated in the promotion of cell survival. They stimulate the phosphorylation of Akt/PKB (1), a serine/threonine kinase that inactivates multiple components of the apoptotic machinery (2-4). The D4 phosphorylated phosphoinositide, phosphatidylinositol 4,5 bisphosphate [PI(4,5)P$_2$ or PIP$_2$], has not been directly shown to promote cell survival, although it may contribute in a number of ways indirectly. PIP$_2$ is a substrate for phosphoinositide 3-kinase that synthesizes the pro-survival D3 lipids (5), and it is a \emph{bona fide} signaling molecule that regulates the actin cytoskeleton, vesicular trafficking, channel and transporter activities and nuclear functions (6). PIP$_2$ synthesis is increased by growth factors (7), by thrombin (8) and by integrin signaling (9). In addition, PIP$_2$ inhibits gelsolin, a caspase substrate (10) that is a major effector of cytoskeletal changes (11). Recently, it was reported that PIP$_2$ complexed with gelsolin inhibits caspase-3 and caspase-9, but not caspase-8 (12), and that PIP$_2$ alone does not inhibit caspases.

We now report that PIP$_2$ alone inhibits initiator and executioner caspases in the two major apoptotic cascades. These cascades start with death receptor activation of procaspase-8 or mitochondrial activation of procaspase-9, and both converge on procaspase-3. We also present \emph{in vivo} evidence for the roles of PIP$_2$ in apoptotic signaling. Human type I phosphatidylinositol phosphate 5 kinase $\alpha$ (PIP5K1$\alpha$) (13-15) protects against apoptosis in both pathways and it is inactivated by caspase-3 cleavage during apoptosis. These results suggest a novel feedforward amplification mechanism for maintaining the balance between phosphoinositide regulation of caspases and caspase regulation of phosphoinositide synthesis.
Experimental Procedures

Lipids-- PIP$_2$ was from either Calbiochem or Boehringer-Mannheim. PI(3,4)P$_2$ and PI(3,4,5)P$_3$ were gifts of C.S. Chen (U. Kentucky). PI(4)P, PS and PC were purchased from Avanti Lipids. Phosphoinositide micelles and mixed vesicles were prepared by probe sonication (16).

Plasmids and recombinant proteins-- Apaf-1 and procaspase-9 were expressed in Sf20 cells and purified (17). Recombinant caspase-3 and caspase-8 were purified from bacteria. PCMV2 procaspase-9 vector was as described in Li et al. (17). The human PIP5K1$\alpha$ cDNA was generated by PCR from a HeLa cell cDNA pool. It was subcloned into pGEM-T (Promega) and an expression vector (pCMV5) containing a myc-tag at the 5’ end (18). The caspase-3 resistant mutant (D279A) and kinase-deficient mutant (D270A) [equivalent to D227 of mouse PIP5K1$\alpha$ (19)] were generated using a QuickChange Site Directed Mutagenesis kit (Stratagene). The PIP5K1$\alpha$ cDNA constructs were subcloned into pET-28c(+) (Novagen) with a hexahistidine tag at the 5’ end. Recombinant PIP5K1$\alpha$ was purified from a Ni-NTA agarose column (Qiagen). Human plasma gelsolin was expressed in $E$. coli and purified by ion exchange chromatography (16). The GFP-AktPH cDNA was a gift of T. Balla (NIH) (20).

Adenovirus containing PIP5K1$\alpha$ or $\beta$-gal were used to infect cells according to protocol in Shibasaki et al. (21).


Caspase activity assays-- Caspase activation in cell extracts: HeLa cell were broken by dounce homogenization in a hypotonic buffer. The lysate was centrifuged at 100,000xg. The high-speed supernatant contained mitochondrial-derived cytochrome c, and the caspase-9 cascade was activated by incubation with 1 mM dATP at 30°C for 1 hr. (22).

Apoptosome activation assay: Pro-caspase-9 was first incubated with PIP₂ micelles on ice for 10 min. in a buffer containing 20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF. Apoptosomes were assembled by mixing procaspase-9 with Apaf-1, cytochrome c (0.01 µg/µl), dATP (100 µM) and MgCl₂ (2.5 mM) and incubating for 1 hr. at 30°C (17, 23).

Fluorogenic caspase activity assay: Enzyme activity was determined by measuring the release of AFC from synthetic substrates at 37°C. Recombinant hamster caspase-3 or human caspase-8 (between 6 to 100 nM) was incubated with 267 µM Ac-DEVD-AFC or Ac-IETD-AFC (Enzyme Systems Products), respectively, in 25 mM Hepes, pH 7.0, 80 mM KCl, 1 mM EGTA. Results were analyzed as described by Zhou et al. (24). Inhibition rates were calculated from progress curves that are generated by adding caspase to a fluorogenic substrate in the presence of 0-20 µM of a phosphoinositide. The rate for the uninhibited reaction (V₀) was obtained from the linear portion of the time course of the reaction, and the rate for the inhibited reaction (Vᵢ) was determined from the steady state formation of the product. The apparent Kᵢₐₚₚ (apparent inhibition constant), was derived from the slope of the [(V₀/Vᵢ) –1] vs PIP₂ curve. Kᵢ, the inhibition constant, was calculated using the equation Kᵢ = (Kᵢ)ₐₚₚ / (1 + [S]/Kₘᵢ), where [S] is the substrate concentration. Kₘᵢ, the Michaelis constant for substrate cleavage, was calculated in the range of 5-200 µM for Ac-IETD-AFC, and of 10-700 µM for Ac-DEVD-AFC using the Lineweaver-Burke plot.
Lipid binding assay-- Mixed lipid vesicles containing 90% PC and 10% of phosphoinositides or PS were prepared by probe sonication in water, and added to caspase-3. The final reaction mixture contained 30 µM of the test phospholipid (phosphoinositide or PS) and 2.5 µM caspase-3 in 20 mM Hepes, pH 7.5, 110 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM PMSF and 0.2 mg/ml BSA. BSA was included to reduce nonspecific binding. After a 30 min. incubation at room temperature, the samples were centrifuged at 100,000g for 30 min. at room temperature. The supernatants were collected and the pellets were resuspended to the original volume. Equivalent volumes were loaded onto SDS gels.

Apoptosis assays-- Transfection: HeLa and HEK293 cells were transfected using Lipofectamine Plus and Lipofectamine (GIBCO), respectively. 2 µg total DNA was used in all cases. Cells were analyzed within 24 hrs. after transfection.

Apoptosis induction: Apoptosis was induced by transfection of pCMV2-FLAG-procaspase-9 (for 24 hrs.), or with apoptotic inducers. These include 1 µM staurosporine or 50 ng/ml TNFα. 10 ng/ml cycloheximide [CHX] or 0.2 µg/ml actinomycin D was added to enhance the apoptotic effect of TNFα. The latter was used in some experiments, because cycloheximide is no longer available commercially. 20 µM z-DEVD-fmk (Enzyme Systems Products) or 200 nM wortmannin (Sigma) was added to cells 30 min. prior to addition of the apoptosis inducers when indicated.

Western blotting: Floating and adherent cells were collected, washed with PBS and lysed with RIPA buffer (50 mM Hepes, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, protease inhibitor cocktail (Boehringer Mannheim), 2 mM phenylmethyl sulfonyle fluoride). In some cases, phosphatase inhibitors (50 mM sodium fluoride, 1 mM orthovanadate, 45 mM pyrophosphate) were included. Samples were subjected to SDS-polyacrylamide gel electrophoresis, and used for western blotting. Endogenous and overexpressed PIP5K1α were detected with an affinity
purified rabbit anti-PIP5K\(\alpha\) antibody (gift of R.A. Anderson, U. Wisconsin) and with anti-c-myc (Santa Cruz), respectively. Other antibodies used are: anti-caspase-3 (Transduction Laboratories), anti-caspase-9 (17) (Pharmingen), anti-Akt and anti-phosphoAkt (New England Biolabs), anti-PARP p85 (Promega), anti-FLAG (Sigma). Immunoreactive bands were detected using the Enhanced Chemiluminescence system (ECL, BioRad).

Microscopy: Apoptotic index was determined using DAPI staining or \(\beta\)-gal staining. For DAPI staining, cells were fixed in formaldehyde and stained with 1 \(\mu\)g/ml DAPI, and with anti-FLAG to detect cells overexpressing procaspase-9. \(\beta\)-gal transfected cells were identified after staining with X-gal. Blue round cells with irregularly shaped nuclei (apoptotic) and blue spread cells (non-apoptotic) in randomly chosen fields were counted in a blinded fashion. More than 500 cells were counted per condition. The two methods gave comparable results.

**PIP5K\(\alpha\) digestion by caspases**-- Cleavage of purified recombinant PIP5K\(\alpha\): PIP5K\(\alpha\) was incubated with caspase or buffer for 60 min. at 37\(^\circ\)C in 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM NaEDTA, 1 mM NaEGTA, 1 mM DTT and 0.1 mM PMSF (caspase-3 buffer).

**In vitro** PIP5K assay: The kinase assay buffer had a final concentration of 1 \(\mu\)Ci [\(\gamma\)-\(32\)P] ATP, 180 \(\mu\)M ATP, 70 \(\mu\)M PI(4)P and a 1:1 w/w ratio of PI(4)P/PS vesicles (13). The reaction was allowed to proceed at 37\(^\circ\)C, and stopped at timed intervals. Samples were extracted with CHCl\(_3\):MeOH:HCl, spotted on thin layer chromatography (TLC) plates together with unlabeled PIP\(_2\) standards. Phospholipids were resolved with 1-propanol:H\(_2\)O:NH\(_2\)OH (65,15,20), and detected by autoradiography. PI(4,5)P\(_2\) standard was visualized with iodine vapor.
Results and Discussion

Effects of PIP₂ on Caspase Activation in Cell Extracts and in in vitro Reconstituted Apoptosomes— The mitochondrial pathway was activated by adding dATP to cell extracts to initiate the Apaf-1/procaspase-9/cytochrome c apoptosome cascade (17,22,23). In untreated cytosolic extracts, procaspase-3 was present as an inactive 32 kDa zymogen (Fig. 1A, left panel, lane 1). dATP generated a 17 kDa band corresponding to the larger caspase-3 subunit, and a decrease in the intensity of the procaspase-3 band (lane 3). Pretreatment of extracts with PIP₂ completely inhibited procaspase-3 processing (lane 2).

Since procaspase-3 is a substrate for caspase-9, the lysates were also blotted with anti-caspase-9 (Fig. 1A, right panel). Procaspase-9 (47 kDa) was detected in the naive lysate (lane 1), and dATP converted all of the procaspase-9 to a 37 kDa mature form. PIP₂ blocked procaspase-9 cleavage, suggesting that PIP₂ acted at the level of the initiator caspase. 3 µM PIP₂/phosphatidylcholine (PC) vesicles were as inhibitory as 3 µM PIP₂ micelles (Fig. 1B, lanes 3 and 4). 3 µM PI(3,4,5)P₃ also decreased pro-caspase-3 cleavage, although to a lesser extent (lane 5). Thus, PIP₂ and PIP₃ inhibit caspase processing, and inhibition occurs in a physiologically relevant milieu.

PIP₂ also inhibited procaspase-9 activation in an in vitro reconstituted apoptosome system (23). In the presence of Apaf-1, cytochrome c and dATP, procaspase-9 was cleaved into two smaller polypeptides (Fig. 1C). Cleavage was dependent on Apaf-1. 5 µM PIP₂ partially inhibited procaspase-9 processing, and 20 µM PIP₂ inhibited it completely. PIP₂ may suppress procaspase-9 activation by inhibiting caspase-9 as soon as it is processed. In this way, further autoprocessing would be blocked. The alternative possibility that PIP₂ inhibits procaspase-9 binding to Apaf-1 is less likely, but has not been ruled out. The instability of
purified recombinant caspase-9 in vitro (25) precluded detailed analysis of the effect of PIP$_2$ on caspase-9 activity.

**Characterization of PIP$_2$ Interaction with Purified Caspases**—PIP$_2$ inhibited purified caspases-8 and -3 in a dose-dependent manner (Fig. 2A and B). We used progress curves to calculate an inhibition constant (K$_i$), according to the method described in Zhou et al. (24). This method is used extensively to estimate the K$_i$ of many inhibitors of apoptosis (26,27). Among the phosphoinositides tested, PIP$_2$ was most potent (Table 1). PIP$_3$ inhibited with a tenfold higher K$_i$ than PIP$_2$. PI(4)P had no effect (Fig. 3A), so its K$_i$ cannot be calculated. IP$_3$, the inositol polyphosphate that is equivalent to PIP$_2$ except that it has no diacylglycerol chain, was not inhibitory at high concentrations (>33 µM, data not shown). Thus, caspases-8 and -3 are able to discriminate between phosphoinositide stereoisomers, and caspase inhibition requires the phosphoinositol headgroup and the diacylglycerol chain. These characteristics are similar to that of some, but not all, phosphoinositide binding proteins (28,29). Since PIP$_2$ is much more abundant than PIP$_3$ (forty-fold by one estimate) (30), PIP$_2$ is likely to be the predominant inhibitor of caspases in quiescent cells, although PIP$_3$ may also directly inhibit caspases in proliferating cells.

The inhibitory effect of PIP$_2$ was reduced by gelsolin, a well-characterized PIP$_2$ binding protein (11,28). This was demonstrated using either caspase-8 or caspase-3. Gelsolin had minimal effect on caspase-8 activity by itself (Fig. 2C). However, gelsolin prevented PIP$_2$ from inhibiting caspase-8. We tested a range of PIP$_2$ concentrations (5-20 µM). We consistently observed reduced caspase-8 inhibition by PIP$_2$ in the presence of gelsolin (data not shown). Likewise, gelsolin blocked the inhibitory effect of PIP$_2$ on caspase-3 (Fig. 2D).

Our results are different from that of Azuma et al. (12) in two respects. First, they found that PIP$_2$ does not inhibit caspase on its own. However, PIP$_2$ becomes inhibitory when
complexed with gelsolin. Second, they reported that although the PIP₂: gelsolin complex inhibits caspase-3 and caspase-9, it did not inhibit caspase-8. Their results suggest that gelsolin may enhance PIP₂ regulation of caspases, while ours indicate that gelsolin competes with caspases for PIP₂. We cannot explain why our results were different. The Azuma group prepared unilamellar lipid vesicles using an extrusion technique (12) and observed no inhibition of caspase activity between 0.25 to 2 μM PIP₂. They did not show results at higher doses. We used PIP₂ from two other sources (Calbiochem and Boehringer-Mannheim), prepared micelles and mixed vesicles by probe sonication. We found a dose-dependent inhibition of caspase activity beginning at 2 μM PIP₂ presented as mixed micelles. Our PIP₂ had no detectible impurities, based on a high pressure liquid chromatography analysis that can distinguish between the PIP₂ from PI(3,4)P₂, PI(4)P and PI(3,4,5)P₃ (D.T. Hilgemann, personal communication). Another potential explanation is that the different assay conditions may affect the outcome. We use a much higher gelsolin: PIP₂ ratio to observe inhibition. Decreasing the gelsolin: PIP₂ ratio however did not promote caspase activity. We have also ruled out that differences in KCl or divalent ion concentration or pH account for our different findings. Although the reason for the discrepancies between the two groups have not yet been resolved, both studies highlight the potential role of PIP₂ in caspase regulation. Competition and cooperation among PIP₂ binding proteins have been documented previously (31), and crosstalk between them may add another level of complexity to their regulation in vivo.

PIP₂ was also inhibitory when presented to caspases in mixed vesicles with PC (Fig. 3A and B). In contrast, 10% phosphatidylinerine (PS)/90% PC vesicles and PI(4)P vesicles were not inhibitory even at high concentrations (Fig. 3A). 2 μM PIP₂ was inhibitory when presented as 4% PIP₂/96% PC vesicles (Fig. 3B). PIP₂ was still inhibitory at even higher dilution (1% PIP₂/99% PC), although more PIP₂ was required. A similar dependence on PIP₂ fractional concentration has been reported for several other PIP₂ binding proteins (32).
Caspase-3 binding to PIP₂ and PIP₃ was demonstrated by cosedimentation with mixed lipid vesicles (Fig. 3C). Consistent with the lack of inhibition by PI(3,4)P₂/PC and PS/PC, caspase-3 did not cosediment with these vesicles. Since caspases do not have recognizable pleckstrin homology domains (PH), which mediate phosphoinositide binding in many proteins (29), their PIP₂ binding domains remain to be identified. PIP₂ binding proteins that do not have a recognizable PH domain often bind PIP₂ through lysine- and arginines-rich regions (33,34).

Protection Against Apoptosis by PIP5Kl Overexpression-- To determine if PIP₂ inhibits caspases in vivo, we overexpressed human type I PIP5Klα (13) by transient overexpression and by adenovirus mediated infection, and examined the effect on apoptosis. Apoptosis was induced by overexpression of procaspase-9 or by treatment with TNFα.

Overexpression of procaspase-9 (17) allowed us to bypass the upstream aspects of apoptotic signaling, and to focus on the effects of PIP₂ on procaspase-9 processing and its downstream sequelae. HEK293 cells were used for transient expression studies, because they can be readily cotransfected with multiple expression vectors at high frequency. Control transfected HEK293 cells had a low level of apoptosis (between 10-14%), and PIP5Klα overexpression had no effect on basal apoptosis. However, PIP5Klα overexpression reduced the percentage of apoptotic procaspase-9 transfected cells significantly (Fig. 4A). Apoptosis was assayed by nuclear condensation as visualized by DAPI staining. Similar results were obtained using morphological criteria upon cotransfection with β-galactosidase (data not shown). In contrast, a kinase-deficient mutant (D270A) (19) did not protect against apoptosis (Fig. 4A), nor did it induce apoptosis in the absence of apoptotic stimuli (data not shown). These results confirm that the kinase activity is required for protection against apoptosis.

PIP5Klα overexpression alters the actin cytoskeleton (18,19), and may also impact the other components of the phosphoinositide cycle. To determine if PIP5Klα overexpression
suppresses apoptosis by inhibiting caspases, we monitored the activation of cotransfected procaspase-9. Western blotting showed that PIP5K1α overexpression increased the ratio of procaspase-9 to caspase-9 from 1.9 to 8.8 (Fig. 4B, right panel). This is consistent with the attenuation of procaspase-9 processing. Moreover, downstream apoptotic events were also suppressed: more procaspase-3 and less caspase-3-digested PARP (the p85 fragment) were recovered. Thus, the apoptotic index and biochemical evidence suggest that PIP5K1α overexpression suppresses the activation of the apical caspase-9, and the downstream effector caspase-3 as well. These results are consistent with protection from apoptosis by PIP2 inhibition of caspases.

Transient overexpression of PIP5K1α in HEK293 cells also protected against apoptosis induced by TNFα (Fig. 4C). Since this death receptor apoptotic cascade is initiated by procaspase-8 activation, and PIP2 inhibits caspase-8 activity in vitro (Fig. 2A), the observed reduction in apoptotic index is likely to be mediated by blocking caspase-8 activation.

In order to estimate how much PIP2 is required to protect against apoptosis, we used adenovirus-mediated infection to introduce PIP5K1α. HeLa cells were used for these studies, because they are efficiently infected by adenovirus (greater than 98%), while HEK293 cells are not. High efficiency infection is required for accurate quantitation of the extent of PIP2 overproduction in the entire cell population. PIP2 synthesis, determined by thin layer chromatography of 32P labeled phospholipids, was increased by 2.6 fold (Fig. 5A). This is accompanied by a decrease in PI(4)P, but not by an increase in PIP3.

This level of PIP2 overproduction protected HeLa cells from TNFα induced apoptosis, as evidenced by the delay in the onset of apoptosis and the extent of apoptosis (Fig. 5B). The 40% percent decrease in apoptosis in adenovirus-infected cells is comparable to that for transiently-transfected HEK293 cells (Fig. 4C). Protection by a moderate level of increased PIP2 production suggests that a physiologically attainable change in the turnover of PIP2 could
affect the progression of apoptosis. In addition, the absence of a detectible increase in PIP3 production suggests that PIP5KIα did not act by increasing PIP3 synthesis.

The Anti-apoptotic Effect of PIP5KIα was not Mediated through PI 3-kinase or Akt Activation---

Although we did not detect an increase in PIP3 synthesis in PIP5KIα overexpressing cells, it is important to use other assays to rule out the possibility that PIP5KIα protects against apoptosis through PIP3 and Akt.

We inhibited PI 3-kinase with wortmannin (Fig. 5B). Wortmannin did not block the anti-apoptotic effect of PIP5KIα. This treatment increased apoptosis in TNFα-treated control-infected cells, but not in PIP5KIα infected cells. Therefore, the anti-apoptotic effect of PIP5KIα was independent of PI 3-kinase.

We monitored Akt activation by assessing Akt phosphorylation and translocation to the plasma membrane. Western blotting with a phospho-Akt specific antibody and a pan-Akt antibody showed that there was no difference in the extent of Akt phosphorylation between β-gal adenovirus and PIP5KIα-adenovirus infected cells (Fig. 5C).

Targeting of Akt to the plasma membrane was monitored by immunofluorescence microscopy. PIP5KIα was cotransfected with the PH domain of Akt tagged to the green fluorescent protein (GFP-AktPH). As shown by others (35), GFP-AktPH is diffusely cytosolic in starved cells, and is recruited to the plasma membrane after insulin stimulation (Fig. 5Di and ii). Pronounced plasma membrane localization of GFP-AktPH is detected in 47.5% of the insulin-treated cells (186 cells counted). Overexpressed PIP5KIα is partly cytosolic, partly punctate and partly plasma membrane associated (Fig. 5Diii) (18). In contrast, GFP-AktPH remains cytosolic in the starved, PIP5KIα overexpressing cell (Fig. 5Div). Only 13.9% of the PIP5KIα overexpressing cells have membrane associated GFP-AktPH, a value indistinguishable from that of cells not overexpressing PIP5KIα (14.3%). Taken together, our data indicate that
PIP5K\(\alpha\) does not protect against apoptosis by activating Akt. Therefore, PIP\(_2\) inhibition of caspases is a more likely primary mechanism for the protection by PIP5K\(\alpha\).

**PIP5K\(\alpha\) Cleavage During Apoptosis**—We noticed that the 68 kDa myc-PIP5K\(\alpha\) band was consistently less intense in cells cotransfected with procaspase-9 (Fig. 4B, left panel), and that two lower molecular weight bands appeared. We therefore investigated the possibility that PIP5K\(\alpha\) is cleaved during apoptosis to generate these fragments. Staurosporine, which activates the mitochondrial apoptotic pathway, decreased the intensity of the full-length myc-PIP5K\(\alpha\) band by 46% (Fig. 6A), and generated a 37 kDa cleavage product. This decrease was not an artifact due to unequal loading, as indicated by the comparable intensity of the \(\alpha\)-tubulin bands. Cleavage of PIP5K\(\alpha\) was prevented by the cell permeant caspase-3 inhibitor z-DEVD-fluoromethylketone (fmk), indicating that it was mediated by caspase-3.

Endogenous PIP5K\(\alpha\) was also degraded during apoptosis. TNF\(\alpha\)/CHX decreased the intensity of the 65 kDa PIP5K\(\alpha\) band by 36% (Fig. 6A). A 37 kDa band was generated, and this band was not observed when apoptosis was blocked with z-DEVD-fmk.

**Caspase-3 Cleavage of PIP5K\(\alpha\)**—The involvement of caspase-3 in PIP5K\(\alpha\) cleavage was confirmed by adding recombinant caspase-3 to HeLa extracts prepared from cells transfected with myc-PIP5K\(\alpha\) and from untransfected cells (Fig. 6B). In both cases, the major product has the same electrophoretic mobility as the band generated in apoptotic cells (Fig. 6A) and cleavage was blocked by z-DEVD-fmk. These results establish that PIP5K\(\alpha\) is cleaved by a caspase-3 dependent pathway during apoptosis. PIP5K\(\alpha\) was cleaved more extensively in the *in vitro* conditions than in apoptotic cells. This may be because in intact cells, only a fraction of the total PIP5K\(\alpha\) is accessible to caspases.
PIP5K\(\alpha\) has a DIPDG sequence (residues 276-280) in its kinase core that conforms to the caspase-3 \(\text{p1DXXD}_{\text{p4}}\) cleavage consensus. We mutated D\(_{279}\) to A to determine if it is part of a \textit{bona fide} caspase-3 cleavage site. At the lowest caspase-3 dose used, wildtype PIP5K\(\alpha\) was already partially cleaved into two major fragments (37 and 28 kDa) (Fig. 7A, lane 2). In contrast, the D279A mutant was resistant to caspase-3 even at a tenfold higher concentration (lane 3). Furthermore, this mutant, when overexpressed in HeLa cells, was not detectably cleaved in staurosporine- or TNF/CHX-treated cells (Fig. 6A).

Caspase cleavage of regulatory proteins often results in a loss-of-function or a gain-of-function (36). PIP5K\(\alpha\) that was partially digested by caspase-3 had decreased kinase activity (Fig. 7B) in an \textit{in vitro} kinase assay. The rate of \(^{32}\text{P}\text{PI(4,5)P}_2\) generation, determined from the slope of the linear portion of the activity curve, was reduced by 50%. The decrease in activity correlates with the 44% cleavage of the PIP5K\(\alpha\) sample used (estimated by densitometry of a western blot similar to that shown in Fig. 7A; actual data not shown). These results establish that caspase-3 cleavage of PIP5K\(\alpha\) causes a loss-of-function. Sequence analyses show that this cleavage site is conserved in the equivalent mouse isoform [mouse PIP5K\(\beta\); note that the nomenclature for the human and mouse isoforms are reversed (15)]. It is not present in the other two known type I PIP5K isoforms, nor is it present in the other major class of phosphoinositide kinases, the type II kinases (37). Modeling from the crystal structure of a type II kinase (37) reveals that the DIPD site in PIP5K\(\alpha\) is likely to be on a solvent-exposed surface that is part of the conserved ATP-binding core of the type I and type II kinases.

\textit{Caspase-3 Cleavage of PIP5K\(\alpha\) Promoted Apoptosis}-- Apoptosis induces the cleavage/inactivation of many proteins involved in signaling cell survival, but the physiological significance of these changes has not been established in most cases. At one extreme, some of these proteins may be merely innocent bystanders that happen to be cleaved during the
execution phase of apoptosis, when the process is already irreversible. To determine if PIP5Kια cleavage is an integral part of apoptotic signaling, we compared the ability of wildtype myc-PIP5Kια and the D279A mutant to suppress procaspase-9 overexpression-induced or TNFα/CHX-induced apoptosis. In each case, apoptosis was suppressed considerably more by the D279 mutant than by the wildtype PIP5Kια (Fig. 4A and C). Western blotting showed that these two isoforms were expressed at comparable levels (Fig. 4D). Therefore, PIP5Kια inactivation by caspase-3 decreases its ability to protect against apoptosis, and this may promote the initiation/progression of the apoptotic cascade.

In conclusion, our results show that PIP2 is a direct inhibitor of initiator and effector caspases. PIP2 inhibits caspases at low absolute concentrations and at high dilutions in mixed lipid vesicles. PIP2 accounts for between 0.4 to 1% of total membrane lipids in cells (30), and PIP2 concentration in the plasma membrane is estimated to be between 4 µM and 10 µM. Local concentrations of PIP2 in membrane may be even higher, since lipids are differentially partitioned in membrane microdomains (38). PIP2 is therefore likely to be present at a high enough concentration to inhibit some caspases in cells. Additional studies will be required to determine how much caspase is associated with PIP2 in non-apoptotic and apoptotic cells. Although early studies suggest that caspases are predominantly cytosolic, recent studies show that some caspases are associated with mitochondrial, microsome or nuclear fractions, and that they redistribute during apoptosis (39-41). Caspase binding to PIP2 may account for some of this association with intracellular organelles, which contain PIP2 (34).

The broad spectrum of caspase inhibition by PIP2 is different from that of previously identified endogenous protein inhibitors of apoptosis (such as XIAP and cIAP) (26,42), and approaches that of the baculovirus protein p35 (24,43), which has no mammalian counterpart. The importance of clamping caspase activity to a minimal level in normal living cells is underscored by the fact that caspase activation triggers a self-amplifying autocatalytic cascade.
and by the existence of multiple checkpoints for caspase activation (36,44). Caspase inhibition may help to establish a threshold for apoptosis and to fine tune the balance between survival and death. The threshold may be lowered by caspase inactivation of PIP5KI\(\alpha\); this would dissipate the pro-survival PIP\(_2\), release the PIP\(_2\) clamp on caspases, and tip the balance toward cell death.
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References


Figure Legends

Figure 1  Effects of PIP2 and PIP3 on procaspase processing in cell-free systems. High speed supernatants of HeLa cell extracts were incubated with dATP to initiate caspase activation and samples were blotted with anti-caspase antibodies. (A) 3 μM PIP2 micelles inhibit procaspase processing. Pro, procaspase; C3, caspase-3, C9, caspase-9. The smaller caspase subunit for each caspase was not detected in cell extracts under these conditions. (B) Extracts were treated with 3 μM each of PIP2 micelles (M), 10% PIP2/PC vesicles (V), and PIP3 micelles and western blotted with anti-caspase-3. (C) Procaspase-9 activation in an in vitro reconstituted apotosome system. Apotosomes were assembled by mixing purified procaspase-9 that was preincubated with 0, 5 or 20 μM PIP2 (lanes 1-3), with Apaf-1, cytochrome C and dATP. Samples were western blotted with anti-procaspase-9.

Figure 2  Effects of PIP2 micelles on caspase-8 and -3 activities. (A) and (B) Representative progress curves for caspase-8 and caspase-3, respectively. Caspases were added to a mixture of tetrapeptide-AFC substrates and PIP2 micelles. AFC generation after substrate cleavage was plotted against time. The different symbols denote PIP2 concentrations used. A, caspase-8 (60 nM). circles, 0 μM; squares, 1 μM; triangles, 3 μM; diamonds, 5 μM; hexagons, 20 μM. B, caspase-3 (30 nM). Symbols (from top to bottom) denote 0, 0.5, 1, 5 and 10 μM. (A’) and (B’), [(V/Vo) –1] was plotted as a function of PIP2 concentration. (C) Gelsolin competed with caspase-8 for PIP2. Caspase-8 (60 nM) was added to solutions containing Ac-IETD-AFC and 10 μM gelsolin preincubated with PIP2 micelles (10 and 20 μM, closed squares and gray diamonds; the two curves overlap) or to PIP2 micelles with no gelsolin (10 and 20 μM, open squares and diamonds). Closed circles indicate control, with caspase-8 and substrate, and no
other addition. Closed triangles indicate caspase-8, substrate and gelsolin in the absence of PIP2. (D) Gelsolin competed with caspase-3 for PIP2. 30 nM caspase-3 was incubated with 4 µM gelsolin and 10 µM PIP2. Symbols are as in (C).

**Figure 3** Effects of PIP2 vesicles on caspase-3 activity. (A) 4 µM 10% PIP2/PC (triangles) inhibited caspase-3, while 20 µM PI (4)P (squares) and PS (10% PS/PC vesicles; diamonds) did not. Circles, caspase-3 in the absence of lipids. (B) PIP2/PC vesicles were inhibitory at low fractional concentrations [1% (open symbols) and 4% (closed triangle) with PC]. Circles, caspase-3 alone; open diamonds, circles and squares, caspase-3 with 0.5, 5 and 10 µM PIP2 in vesicles containing 1% PIP2 and 99% PC. Closed triangle, caspase-3 with 2 µM PIP2 in vesicles containing 4% PIP2 and 96% PC. (C) Caspase-3 binding to lipid vesicles. Mixed lipid vesicles containing 90% PC, and 10% of one of the following: PI(3,4,5)P3, PI(4,5)P2, PI(3,4)P2, PS (lanes 1-4, respectively, at a final concentration of 30 µM) were incubated with 2.5 µM caspase-3 and collected by high speed centrifugation. Equivalent fractions of supernatants and pellets were analyzed by SDS-polyacrylamide gel electrophoresis and Coomassie blue staining.

**Figure 4** PIP5Kια overexpression protects against apoptosis. (A and B) Effects of PIP5Kια on apoptosis in procaspase-9 overexpressing cells. (A) HEK293 cells were transfected with blank or PIP5Kια-containing vectors and procaspase-9 (3:1 weight ratio). Twenty four hours later, they were stained with anti-FLAG (to detect procaspase-9 expression) and with DAPI. Procaspase-9 overexpressing cells that were apoptotic were scored by the presence of condensed DAPI-stained nuclei. Background apoptosis observed in cells not transfected with procaspase-9 (between 10-15%) was subtracted, and the values shown are mean+/−SEM of three independent experiments. D279A is the caspase-3 resistant mutant, and D270A is the kinase deficient mutant. Overexpression of the wildtype (wt) or mutant PIP5Kια had no detectible effect on apoptosis in the absence of apoptotic stimuli (data not shown). (B) Western
blotting. Lanes 1, lysate from HEK293 cells transfected with procaspase-9; lanes 2, procaspase-9 and PIP5K\(\alpha\); lane 3, PIP5K\(\alpha\) alone. C-9, caspase-9; C-3, caspase-3. There was no detectible variation in protein loading, based on the equivalent intensity of beta-tubulin band in these lanes (data not shown). The result shown is representative of two independent experiments. (C) Effects of wt and D279A PIP5K\(\alpha\) overexpression on apoptosis induced by treatment with TNF\(\alpha\)/CHX for 3 hrs. PIP5K\(\alpha\) and \(\beta\)-gal cDNAs were cotransfected at a 2:1 weight ratio. Left panel, transfected HEK293 cells that expressed \(\beta\)-gal were detected with X-gal, and examined for morphological signs of apoptosis. Data shown is the mean+/−SEM of two independent experiments.

**Figure 5** Effect of PIP5K\(\alpha\) overexpression on PIP\(_3\) synthesis and Akt activation. (A) \(^{32}\)P incorporation into phospholipids, analyzed by thin layer chromatography. HeLa cells were infected with PIP5K\(\alpha\) or \(\beta\)-gal adenovirus and labeled with \(^{32}\)P. Phospholipid standards were used to identify the \(^{32}\)P labeled lipids. (B) HeLa cells infected with PIP5K\(\alpha\) or \(\beta\)-gal adenovirus were treated with 200 nM wortmannin or carrier (DMSO) for 30 min. at 37°C. They were then exposed to TNF\(\alpha\)/actinomycin D for 1.5 or 3 hrs. Cells were stained with DAPI, and apoptotic nuclei were scored. About 500 cells were counted for each condition per experiment. Data shown is mean+/−SEM of two independent experiments. (C) Western blotting for phospho-Akt after infection of Hela cells with \(\beta\)-gal or PIP5K\(\alpha\) adenovirus. Cells were serum-deprived in 0.5% fetal calf serum overnight, lysed in the presence of phosphatase inhibitors, and western blotted with anti-Akt and anti-phospho-Akt. (D) Immunofluorescence localization of GFP-AktPH after insulin stimulation or coexpression with myc-PIP5K\(\alpha\). HEK293 cells were transfected with 0.5 \(\mu\)g GFP-AktPH either alone or with 0.5 \(\mu\)g myc-PIP5K\(\alpha\) cDNA, and serum-deprived overnight. (Di and Dii) localization of GFP-AktPH without and with 1 \(\mu\)M insulin for 10 min.,
respectively. Cells were not overexpressing PIP5K1α. (Diii and Div) localization of myc-PIP5K1α and GFP-AktPH, respectively, in a starved cell cotransfected with both cDNAs.

**Figure 6** PIP5K1α is a caspase-3 substrate *in vivo* and *in vitro*. (A) Cleavage of overexpressed and endogenous myc-PIP5K1α. Left panel, HeLa cells transfected with wildtype or caspase-3 resistant (D279A) myc-PIP5K1α were treated with staurosporine for 6 hrs. in the absence or presence of z-DEVD-fmk. Cell lysates were analyzed by western blotting with anti-myc and anti-α tubulin. Right panel, HeLa cells (not transfected) were treated with TNFα/CHX for 6 hrs. Endogenous PIP5K1α was detected with affinity-purified anti-PIP5K1α. (B) Cleavage of overexpressed and endogenous PIP5K1α by caspase-3 in cell extracts. Extracts prepared from cells transfected with myc-PIP5K1α and from untransfected HeLa cells were incubated with recombinant caspase-3 for 60 min. at 37°C, in the presence or absence of 50 µM z-DEVD-fmk.

**Figure 7** Caspase-3 cleaves and inactivates PIP5K1α. (A) Identification of the caspase-3 cleavage site. Purified, recombinant his-tagged wildtype (wt) and D279A PIP5K1α were incubated with caspase-3 for 60 min. at 37°C, and western blotted with anti-PIP5K1α. Lanes 1, untreated hPIP5K1α; lanes 2 and 3, with caspase-3, tenfold higher amount in lane 3 than in lane 2; lane 4, same amount of caspase-3 as in lane 3, but with no PIP5K1α. (B) *In vitro* kinase assay. PIP5K1α was incubated with caspase-3 or buffer for 60 min. at 37°C. Digestion of PIP5K1α was monitored by western blotting (not shown). The digested and undigested kinase were incubated with PI(4)P and [γ-32P]ATP at 37°C and reaction was stopped at timed intervals. 32P-labeled PIP2 was detected by autoradiography of a thin layer chromatogram (top panel). Radioactivity associated with PIP2 was determined by scintillation counting, and plotted as a
function of time (bottom panel). Open circles, without (w/o) caspase-3; closed circles, with (w/) caspase-3. Results shown are representative of three independent determinations.
### Table 1. Inhibition Constants ($K_i$) for Caspases

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Regulation of Apoptosis by PIP2 and PIP5K

Mejillano . . . Yin

Fig. 1A-C

**A. Caspase-3 vs. Caspase-9**

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Caspase-3

- Pro-C3
- C3 (p17)

Caspase-9

- Pro-C9
- C9 (p37)

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Pro-C3

- C3 (p17)

**C.**

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Pro-C9

- C9 (p37)

C9 (p10)
Regulation of Apoptosis by PIP2 and PIP5K

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Fig. 2A-D

A. 

B. 

C. 

D.
Fig. 3A-B
A. Caspase-9

![Bar graph showing % Apoptosis for Control, wt, D279A, and D270A with error bars.]

B. Western blot analysis

- Lane 1: wt, no treatment
- Lane 2: D279A, no treatment
- Lane 3: D270A, no treatment

- Lane 1: PARP (p85)
- Lane 2: pro-C9
- Lane 3: C9

C. TNFα/CHX

![Bar graph showing % Apoptosis for Control, wt, and D279A with error bars.]

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Fig. 4A-C
Regulation of Apoptosis by PIP$_2$ and PIP5K

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Fig. 5A-D
A. in vivo

Staurosporine
myc-PIP5K1α
1  2  3  1'  2'

62
32.5

Stimulus
z-DEVD-fmk - + + +

- + + +

anti-myc

TNF/CHX
endogenous PIP5K1α
1  2  3

- + + +

- + + +

α-tubulin

anti-myc

anti-PIP5K1α

B. in vitro

myc-PIP5K1α
1  2  3  4

62
32.5

C-3
z-DEVD-fmk

- + + +

- + + +

Extract

+ + + +

+ + + +

anti-myc

anti-PIP5K1α
Fig. 7A-B

A. PIP5K\(\alpha\)

B. w/ C-3  w/o C-3

Radioactivity, cpm

Time (min)
Regulation of apoptosis by phosphatidylinositol 4, 5 Bisphosphate inhibition of caspases, and caspase inactivation of phosphatidylinositol phosphate 5 kinases
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