Chapter 2

PtdIns(4,5)P₂ depletion is essential for stress-induced apoptosis

submitted
PtdIns(4,5)P$_2$ depletion is essential for stress-induced apoptosis

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Stress-induced apoptosis is believed to contribute to a number of human diseases however, little is known about the signalling events that regulate this process. Reports demonstrating that the phosphoinositide, phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P$_2$) inhibits caspase activation and promotes pro-survival signals has led to the hypothesis that PtdIns(4,5)P$_2$ suppresses apoptosis. However, to date it has not been demonstrated that apoptotic stimuli negatively regulate PtdIns(4,5)P$_2$ levels. Here, we show that both hydrogen peroxide (H$_2$O$_2$) and UV-irradiation, two apoptotic stress stimuli, cause irreversible depletion of PtdIns(4,5)P$_2$ in a caspase-independent manner. Depletion of PtdIns(4,5)P$_2$ is essential, as ectopic expression of phosphatidylinositol 4-phosphate 5-kinase (PIP 5-K), a lipid kinase which synthesises PtdIns(4,5)P$_2$ in vivo, rescues cells from H$_2$O$_2$-induced apoptosis. We find that H$_2$O$_2$ inhibits PIP 5-K activity and simultaneously induces the translocation of PIP 5-K away from its substrate at the plasma membrane. These observations identify PtdIns(4,5)P$_2$ as an essential regulator of stress-induced apoptosis and establishes PIP 5-K as a target for control by stress stimuli.

Introduction
Stress-induced apoptosis (or programmed cell death) is linked to the aetiology of a number of pathological conditions. For example, both the onset of Alzheimer’s disease and cardiac infarction have been linked to apoptosis induced by reactive oxygen species, such as H$_2$O$_2$ (Andersen, 2004; Zhao, 2004). A key survival pathway in cells inhibiting multiple components of the apoptotic machinery is the PKB/Akt signalling cascade. PKB is activated by PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ which clearly implicates these 3-phosphorylated phosphoinositides in the promotion of cell survival (Alessi, 1996; Stokoe, 1997; Scheid 2003). In contrast, the role of the phosphoinositide, PtdIns(4,5)P$_2$, in promoting cell survival remains less well defined. PtdIns(4,5)P$_2$ regulates a wide range of cellular processes, including ion channel activation, actin cytoskeleton remodelling and vesicular trafficking (Yin, 2003; Itoh, 2004; Hilgemann, 2004). PtdIns(4,5)P$_2$ is also hydrolysed by
phospholipase C (PLC) generating the second messengers, diacylglycerol (DAG) which promotes cellular proliferation (via protein kinase C activity) and IP3 which regulates intracellular calcium release. PtdIns(4,5)P2, is synthesised in vivo by the phosphorylation of PtdIns4P on the 5 position of the inositol head group by the PIP 5-K family of lipid kinases. The PIP 5-K family is encoded by four genes, which generate a number of different protein products that appear to have non-redundant functions (Loijens, J.C, 1996; Ishihara, 1998). We have shown that in response to H2O2, PIP 5-K synthesises PtdIns(3,4,5)P3 via its PtdIns(3,4)P2 5-kinase activity (Halstead, 2001). This data implicates PIP 5-K in cell survival, as activation of the anti-apoptotic protein kinase, PKB/Akt is PtdIns(3,4,5)P3-dependent. A more direct role for PIP 5-K and PtdIns(4,5)P2 in the regulation of cell survival has been suggested as PtdIns(4,5)P2 can inhibit the activation of caspases 8, 9 and 3 (Mejillano, 2001; Azuma, 2000). A possible link between caspase activation and PtdIns(4,5)P2 levels comes from the observation that human PIP 5-Kα (the homologue of murine PIP 5-Kβ) is cleaved and inactivated by caspases (Mejillano, 2001). In this model of apoptotic control, activated caspases cleave and inhibit PIP 5-K thereby blocking PtdIns(4,5)P2 synthesis and triggering further caspase activation.

In cardiomyocytes, a role for PtdIns(4,5)P2 depletion has been suggested in promoting apoptosis. Expression of a constitutively active version of the alpha subunit of Gq (Gqa*) ectopically expressed in HeLa cells to deplete PtdIns(4,5)P2 levels. The GFP fusion of the PH domain of PLCβ1 (GFP-PHPLC) was used as an in vivo PtdIns(4,5)P2 probe (Varnai, 1998; Stauffer, 1998). GFP-PHPLC is concentrated at the plasma membrane in control cells (not expressing Gqa*)(Figure 1A). In HeLa cells expressing Gqa*, GFP-PHPLC was spread diffusely throughout the cell (with an apparent membrane-cytosolic ratio of 1:1) (Figure 1B), indicating that plasma membrane PtdIns(4,5)P2 levels were

Results

Sustained PtdIns(4,5)P2 depletion leads to apoptosis.

As a first step towards clarifying the role of PtdIns(4,5)P2 in apoptosis, we used a constitutively active version of the alpha subunit of Gq (Gqa*) ectopically expressed in HeLa cells to deplete PtdIns(4,5)P2 levels. The GFP fusion of the PH domain of PLCβ1 (GFP-PHPLC) was used as an in vivo PtdIns(4,5)P2 probe (Varnai, 1998; Stauffer, 1998). GFP-PHPLC is concentrated at the plasma membrane in control cells (not expressing Gqa*)(Figure 1A). In HeLa cells expressing Gqa*, GFP-PHPLC was spread diffusely throughout the cell (with an apparent membrane-cytosolic ratio of 1:1) (Figure 1B), indicating that plasma membrane PtdIns(4,5)P2 levels were
Figure 1, Expression of Gaq* in HeLa cells depletes PtdIns(4,5)P₂. HeLa cells were grown overnight on cover slips and then transfected with GFP-PHₚlc alone or together with Gaq*. GFP-PHₚlc localisation was then examined by confocal microscopy 24 hours after transfection. Shown are representative images of the PtdIns(4,5)P₂ probe transfected alone (A) or co-transfected with Gaq* (B). (C) HeLa cells were grown overnight on glass cover slips and then transfected with GFP-Histone or with both GFP-Histone and Gaq*. 36 hrs after transfection cells were fixed, permeabilised and stained with TOPRO-Red to visualise nuclei. Cells were then analysed via confocal imaging. Shown are representative images of GFP and TOPRO-Red channels from cells expressing both GFP-Histone and Gaq*. (D) PtdIns(4,5)P₂ labelling is increased in cells expressing murine PIP 5-Kα. HeLa cells were transfected with either GFP Histone (designated as control on graph) or GFP-PIP 5-K (designated as PIP 5-K on graph). Cells were then [³²P]-orthophosphate labelled, after which phospholipids were extracted and analysed by TLC. Plotted are the resulting PtdIns(4,5)P₂ levels. Error bars display standard deviation of triplicate samples. This graph is typical of three independent experiments. (E) PIP 5-Kα attenuates Gaq*-induced apoptosis. HeLa cells were transfected with the constructs shown. For these experiments a myc-tagged PIP 5-K construct was used. Cells were left for 36 hrs, collected, fixed and stained with Hoechst 33258. Cells were then examined for apoptosis (as defined by nuclear fragmentation). Only transfected cells that were GFP positive were scored. Typically, a total of 500 cells were counted for each sample. Mean values of duplicate samples are plotted. Shown is a graph representative of three independent experiments.

markedly depleted. In line with previous data from cardiomyocytes, expression of Gaq* caused apoptosis in HeLa cells in a caspase-dependent manner (Howes, 2003) (Figure 1C and Figure 1E). A causal link between Gaq*-mediated PtdIns(4,5)P₂ depletion and apoptosis has never been made and to test this point, we sought to inhibit cell death by rescuing PtdIns(4,5)P₂ levels. It has been previously reported by our group and others that the expression of PIP 5-K isoforms elevates PtdIns(4,5)P₂ levels by 1.5 – 2 fold in the majority of adherent cell lines (this figure is based upon 70 % of a cell population ectopically expressing PIP 5-K) and indeed this is also the case in HeLa cells (Figure 1D). Therefore we attempted to rescue
Figure 2. H$_2$O$_2$ causes apoptosis in a caspase-dependent manner. (A) HeLa cells were transfected with a GFP Histone. Cells were stimulated with 600 μM H$_2$O$_2$ in the presence or absence of ZVAD-fmk as indicated for 24 hr and fixed. The nuclei of cells were then stained with Hoechst 33258 and examined using fluorescence microscopy to identify transfected cells that displayed fragmented apoptotic nuclei. Plotted graphically are the mean values of duplicate samples. The number of apoptotic cells displaying fragmented nuclei is plotted as a percentage of the total number of transfected cells. Typically, a total of 500 cells were counted for each sample. The data shown are representative of three different experiments. (B) H$_2$O$_2$ induces PtdIns(4,5)P$_2$ depletion in HeLa cells. Cells were grown overnight on glass coverslips and transfected with GFP-PH$_{PLC}$. Cells were stimulated with 600 μM H$_2$O$_2$ and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation (0 min) with H$_2$O$_2$ and 60 min after stimulation with H$_2$O$_2$. (C) PtdIns(4,5)P$_2$ depletion by H$_2$O$_2$ can be visualised using FRET. Cells were transfected with YFP and CFP chimeras of the PH domain from PLCβ1. FRET changes were followed on a wide-field microscope by calculating the ratio of the CFP and YFP fluorescence (Van der Wal, 2001). Prior to stimulation the responsiveness of each cell was assessed by NKA addition. Cells were then treated with 600 μM H$_2$O$_2$ and the fluorescence ratio was monitored. 45 minutes post stimulation ionomycin and Ca$^{2+}$ were added. The points of NKA, H$_2$O$_2$, ionomycin and Ca$^{2+}$ addition are indicated. Shown is a representative FRET trace of many independent experiments. (D) H$_2$O$_2$ induces changes in phosphoinositide metabolism. Cells were labelled with [32P]-orthophosphate for two hours. They were stimulated with H$_2$O$_2$, for the time indicated and the phospholipids extracted from the cells. Lipid samples were then deacylated and the resulting glycerophosphoinositides separated on a PEI-cellulose plate. The positions of gPIns(4,5)P$_2$, gPIns(3,4)P$_2$, and gPIns(3,4,5)P$_3$ are indicated. (E) H$_2$O$_2$ induces transient synthesis of PtdIns(3,4,5)P$_3$. HeLa cells expressing mRFP-PH$^{grp1}$ were treated with 600 μM H$_2$O$_2$ and confocal images taken every minute for 60 min. Shown are representative images of cells prior to stimulation with H$_2$O$_2$, 10 min and 30 min after stimulation with H$_2$O$_2$. (F) Transient PtdIns(3,4,5)P$_3$ production by H$_2$O$_2$ can be visualised using a FRET based assay. Cells expressing both GFP-PH$^{grp1}$ and mRFP-PH$^{grp1}$ were treated with 600 μM H$_2$O$_2$ and FRET changes were followed on a wide-field microscope by calculating the ratio of the GFP and mRFP fluorescence.
PtdIns(4,5)P$_2$ levels in HeLa cells by co-expressing murine PIP 5-K$. In support of the idea that PtdIns(4,5)P$_2$ depletion is a critical step during Gq*-induced apoptosis, expression of PIP 5-K$ potently blocked cell death (Figure 1E).

**Apoptotic stress stimuli cause sustained PtdIns(4,5)P$_2$ depletion.**

Having demonstrated that PtdIns(4,5)P$_2$ depletion is essential for Gq*-mediated apoptosis, the study was broadened to test whether other apoptotic stimuli also negatively regulate PtdIns(4,5)P$_2$ levels.

Therefore, we began by assessing H$_2$O$_2$-induced apoptosis in HeLa cells. It is important to note that H$_2$O$_2$ influences cell behaviour in a concentration-dependent manner as higher concentrations cause necrosis while lower concentrations cause cell cycle arrest and apoptosis (Finkel, 2003). It was therefore critical to establish a concentration of H$_2$O$_2$ that induced apoptosis rather than necrotic cell death. In our cell system, 600 $\mu$M H$_2$O$_2$ reproducibly caused apoptosis, which was potently blocked by the caspase inhibitor, ZVAD-fmk (Figure 2A).

![Figure 3](image-url)

**Figure 3, UV-irradiation induces PtdIns(4,5)P$_2$ depletion.** (A) HeLa cells were grown overnight on glass coverslips. They were then transfected with GFP-PH$^{PLC}$. Using confocal imaging, living cells were recorded for 2-3 min and then stimulated with UV-irradiation for 30 seconds. By closing the field diaphragm only the centre cells (within the white circle) were exposed to UV-irradiation. Cells were then imaged for 10 min with images being recorded every 30 seconds. Shown are representative images of cells prior to stimulation and 10 mins after UV-irradiation. (B) The cartoon highlights two analysed cells for which the intensities of the fluorescence of the membrane (red line), and of the cytosol (blue line), and the membrane/cytosol ratio (black line) were plotted in time. The graphs for each cell is indicated via a dashed line. Note the drop in the membrane/cytosol ratio of the cell outside the circle (indicated by the dotted line) after UV-irradiation and that the membrane/cytosol ratio of the cell outside the circle remains constant. Scale bar signal shows percent deviation from baseline ratio value. Time is also indicated, as is the point of UV-irradiation. (C) H$_2$O$_2$ induces PtdIns(4,5)P$_2$ depletion in a caspase-independent manner. HeLa cells were grown overnight on glass coverslips. They were then transfected with a GFP-PH$^{PLC}$ construct. 16 hours post-transfection, cells were incubated with 50 $\mu$M (final concentration) ZVAD-fmk for one hour. Cells were then treated with 600 $\mu$M H$_2$O$_2$ and the cells were imaged for 30 minutes with images being taken every minute. (D) Same as in (C) however cells were stimulated with UV-irradiation for 30 seconds. Cells were then imaged for 10 mins with images being recorded every 30 seconds. Shown are representative images prior to treatment and after treatment with H$_2$O$_2$ or UV-irradiation (as indicated).
Cells were stimulated with 600 μM H$_2$O$_2$ and the localisation of GFP-PH$^{PLC}$ was monitored using live confocal imaging techniques. H$_2$O$_2$ caused translocation of GFP-PH$^{PLC}$ from the plasma membrane indicative of sustained PtdIns(4,5)P$_2$ depletion (Figure 2B). Even after prolonged stimulation with H$_2$O$_2$, PtdIns(4,5)P$_2$ levels at the plasma membrane did not recover, as GFP-PH$^{PLC}$ remained diffusely spread throughout the cell. To monitor PtdIns(4,5)P$_2$ depletion with increased temporal resolution, we used a FRET based assay (Van der Wal, 2001). During each experiment, cells were stimulated with neurokinin A to test the responsiveness of individual cells to agonist-induced PtdIns(4,5)P$_2$ changes. NKA mediated stimulation of PLC activity caused a transient decrease in PtdIns(4,5)P$_2$ which recovered to near basal levels within 2 minutes. Conversely, H$_2$O$_2$ treatment caused a sustained decrease in the FRET ratio, indicating prolonged and sustained PtdIns(4,5)P$_2$ depletion. Complete activation of PLC by the addition of ionomycin and extracellular Ca$^{2+}$ did not elicit further decreases in the FRET ratio indicating that plasma membrane PtdIns(4,5)P$_2$ had already been depleted (Figure 2C).

To support our confocal studies, we sought to demonstrate PtdIns(4,5)P$_2$ depletion by an independent method. Therefore we labelled cells with $^{32}$P-orthophosphate and monitored phosphoinositide levels during a time course of H$_2$O$_2$ treatment. H$_2$O$_2$ markedly decreased PtdIns(4,5)P$_2$ labelling compared to untreated controls (Figure 2D). The labelling data was in good agreement with the FRET and confocal data, indicating that apoptotic concentrations of H$_2$O$_2$ negatively regulate PtdIns(4,5)P$_2$ levels. Moreover, this was a rapid process as PtdIns(4,5)P$_2$ labelling was significantly depleted within twenty minutes of H$_2$O$_2$ treatment. As previously observed by our group and others, both PtdIns(3,4,5)P$_3$ and PtdIns(3,4)P$_2$ labelling were increased after H$_2$O$_2$ treatment (Van der Kaay, 1999; Halstead, 2001) (Figure 2D). The increase in PtdIns(3,4,5)P$_3$ labelling however was clearly transient. Intriguingly, the increase in PtdIns(3,4)P$_2$ labelling was sustained after H$_2$O$_2$ treatment suggesting that synthesis of this lipid occurs in the absence of observable PtdIns(3,4, 5)P$_3$ levels. The transient production of PtdIns(3,4,5)P$_3$ inferred by our labelling studies was confirmed with live cell imaging in which PtdIns(3,4,5)P$_3$ levels were monitored after H$_2$O$_2$ treatment in real-time using a monomeric RFP fusion of the PH domain of Grp1 (mRFP-PH$^{Grp1}$) as an in vivo PtdIns(3,4,5)P$_3$ probe (Gray, 1999). This probe, which is predominantly cytosolic was transiently recruited to the plasma membrane after H$_2$O$_2$ treatment (Figure 2E). The transient synthesis of PtdIns(3,4,5)P$_3$ production after treatment with H$_2$O$_2$ was further confirmed by a FRET based assay in which mRFP-PH$^{Grp1}$ and GFP-PH$^{Grp1}$ were used as a FRET pair to follow PtdIns(3,4,5)P$_3$ production with improved temporal resolution (Figure 2F).

To examine whether depletion of PtdIns(4,5)P$_2$ occurred with other apoptotic stress stimuli, HeLa cells expressing GFP-PH$^{PLC}$ were stimulated with UV-irradiation and monitored using confocal microscopy. UV-irradiation also triggered translocation of GFP-PH$^{PLC}$ from the plasma membrane to the cytosol (Figure 3A and Figure 3B). The time-scale of the translocation of GFP-PH$^{PLC}$ after UV treatment was also rapid occurring within 10 min. Together, these data demonstrate that two apoptotic stress stimuli trigger sustained decreases in
PtdIns(4,5)P₂ levels.

PtdIns(4,5)P₂ depletion is essential for stress induced apoptosis.

The in vivo synthesis of PtdIns(4,5)P₂ is dependent upon the PIP 5-K family of lipid kinases and it is possible that the observed decreases in PtdIns(4,5)P₂ resulted from inactivation of PIP 5-K activity. As apoptosis by H₂O₂ and UV-irradiation are both caspase dependent and one isoform of human PIP 5-K is cleaved and inactivated by caspases, we considered the possibility that PtdIns(4,5)P₂ depletion may occur downstream of caspase activation. While pre-incubation of HeLa cells with the general caspase inhibitor (ZVAD-fmk) attenuated H₂O₂ induced apoptosis (Figure 2A), ZVAD-fmk, failed to block depletion of PtdIns(4,5)P₂ induced by either H₂O₂ (Figure 3C) or UV-irradiation (Figure 3D). These data demonstrate that apoptotic stress stimuli deplete PtdIns(4,5)P₂ levels in a caspase-independent manner.

Our data indicated that PtdIns(4,5)P₂ depletion was an essential step during Gαq*-dependent apoptosis. To determine if H₂O₂-dependent apoptosis was also reliant upon PtdIns(4,5)P₂ depletion, we sought to block cell death by expressing PIP 5-Kα. Importantly, we found that ectopic expression of PIP 5-Kα completely blocked apoptosis by H₂O₂ in HeLa cells (Fig. 4a). This block in H₂O₂-induced apoptosis was dependent upon the lipid products of PIP 5-Kα, as a ‘kinase dead’ version of PIP 5-Kα
(that retains 1% residual activity compared to the wild-type enzyme) offered negligible protection against H₂O₂-induced apoptosis (data not shown). Furthermore, the rescue of cells from apoptosis was not limited to H₂O₂ treatment, as PIP 5-Kα also rescued HeLa cells from UV-irradiation-induced apoptosis (Fig. 4b). As PIP 5-Kα synthesises PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ in vivo (Halstead, 2001), we examined if the protective effect of PIP 5-K was linked to the activation of PKB. Cells were transfected with PIP 5-Kα or a constitutively activated PI 3-kinase (CAAX PI 3-kinase) in the absence or presence of PKB and PKB activity was assessed. While the CAAX PI 3-kinase potently activated PKB, no such stimulation was observed with PIP 5-Kα (Figure 5A). This result was confirmed by examining endogenous PKB phosphorylation at Serine⁴⁷³ and Threonine³⁰⁸. Insulin, EGF and CAAX PI 3-kinase were potent activators of PKB however PIP 5-Kα failed to promote phosphorylation of either amino acid (Figure 5B). As H₂O₂ triggered PtdIns(4,5)P₂ depletion, it was possible that the levels of PtdIns(4,5)P₂ required for PtdIns(3,4,5)P₃ synthesis were maintained by PIP 5-Kα expression. H₂O₂ transiently activated PKB, as Ser⁴⁷³ phosphorylation was lost after 60 minutes (Figure 5C). Importantly, ectopic expression of PIP 5-Kα failed to prolong PKB Ser⁴⁷³ phosphorylation upon long-term H₂O₂ treatment (Figure 5D). Furthermore, it is unlikely that PIP 5-Kα-mediated PtdIns(3,4,5)P₃ synthesis attenuated H₂O₂-induced apoptosis, as the PI 3-kinase inhibitor LY294002 (Vlahos, 1994), which blocks the ability of PIP 5-Kα to generate PtdIns(3,4,5)P₃, had no effect upon the PIP 5-K-dependent rescue of cells after H₂O₂ treatment (data not shown). Together, these data indicated that PIP 5-K-dependent rescue from apoptosis was dependent upon PtdIns(4,5)P₂ and not PtdIns(3,4,5)P₃; and that PtdIns(4,5)P₂ depletion is an essential step during H₂O₂ and UV-irradiation-induced apoptosis.

**Apoptotic stress stimuli attenuate PIP 5-K activity during apoptosis**

As there was a close temporal correlation between the labelling decreases in both PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ after H₂O₂ treatment (production of both these lipids is dependent upon PIP 5-K activity), we considered the possibility that PIP 5-K activity was inhibited after prolonged oxidative stress. To test this, we assayed endogenous PIP 5-K activity from HeLa cells after in vivo treatment with 600 µM H₂O₂. Endogenous PIP 5-K activity was inhibited after 20 minutes incubation with H₂O₂ (Figure 6A). The inhibition was transient as PIP 5-K activity recovered with time, demonstrating that PIP 5-K inhibition by apoptotic concentrations of H₂O₂ was reversible. As H₂O₂ inactivated PIP 5-K in a transient manner, this mechanism was unlikely to fully explain the sustained depletion of PtdIns(4,5)P₂ levels we observed by confocal and labelling studies. Therefore, we examined other potential mechanisms for the down-regulation of PIP 5-K activity. It is known that PIP 5-K activity is abundant in membrane fractions and PIP 5-K localises to membranous structures and is enriched at the plasma membrane (Figure 6B). As the PIP 5-K substrate PtdIns4P is membrane localised and a number of reports have linked in vivo PIP 5-K activity to membrane localisation (Kunz, 2000; Kunz, 2002), we tested whether treatment with apoptotic concentrations of H₂O₂ altered PIP 5-K localisation. Indeed, within minutes H₂O₂ treatment, a GFP fusion of PIP5-Kα (GFP-PIP 5-Kα) translocated from the plasma membrane to the cytoplasm in a manner reminiscent of the H₂O₂- induced trans-
location of GFP-PH$^{PLC}$ (Figure 6B). The translocation of GFP-PIP 5-K$\alpha$ was also observed after UV-irradiation (Figure 6C). In both cases, we were unable to detect enrichment of GFP PIP 5-K$\alpha$ at the plasma membrane several hours after treatment. It has been shown previously that PIP 5-K mutants which localise to the cytosol do not efficiently elevate PtdIns(4,5)P$_2$ levels in vivo (Kunz, 2000; Kunz, 2002). The translocation of the enzyme from the plasma membrane could therefore constitute a primary mechanism by which PtdIns(4,5)P$_2$ synthesis at the membrane could be attenuated. As GFP PIP 5-K$\alpha$ translocation after H$_2$O$_2$ treatment correlated with a decrease in PtdIns(4,5)P$_2$, we tested whether sustained PtdIns(4,5)P$_2$ hydrolysis would alter GFP PIP 5-K localisation. Cells were co-transfected with GFP PIP 5-K$\alpha$ and a mutated version of the neurokinin A (NKA) receptor, which is not desensitised after stimulation, leading to prolonged PLC activation and sustained PtdIns(4,5)P$_2$ hydrolysis (Alblas, 1995). To observe changes in both GFP-PIP 5-K localisation...
and PtdIns(4,5)P\textsubscript{2} levels, we used a monomeric red fluorescent protein fusion of the PH domain of PLC\textsubscript{G1} (mRFP-PH\textsuperscript{PLC}). After NKA stimulation, mRFP-PH\textsuperscript{PLC} translocated from the plasma membrane to the cytosol within 20 seconds (Figure 6D). GFP PIP 5-K\textalpha\textbeta however remained localised at the plasma membrane indicating that depletion of PtdIns(4,5)P\textsubscript{2} alone, or activation of downstream targets of the PLC pathway, was insufficient to cause translocation of the lipid kinase. If the translocation of PIP 5-K from the plasma membrane was important for the observed loss of PtdIns(4,5)P\textsubscript{2} levels it would be expected that the translocation of the lipid kinase would precede the translocation of the \textit{in vivo} PtdIns(4,5)P\textsubscript{2} probe. In support of this idea, we observed translocation of GFP PIP 5-K\textalpha in HeLa cells prior to

**Figure 6, Endogenous PIP 5-K is catalytically inactivated \textit{in-vivo} after treatment with H\textsubscript{2}O\textsubscript{2}.** (A) HeLa cells were stimulated for the time indicated. Cells were lysed and endogenous PIP 5-kinase was immunoprecipitated using a polyclonal antibody. Immunoprecipitates were tested for associated PIP 5-kinase activity using a mixed micelle containing PtdIns(4)P as a substrate (see materials and methods). Samples were extracted and the lipids resolved on using TLC. Plotted graphically are the mean values of triplicate samples. Error bars represent the standard deviation of the mean of triplicate samples. (B) H\textsubscript{2}O\textsubscript{2} induces delocalisation of PIP 5-K from the plasma membrane. Shown are confocal images of the mid-section of cells expressing GFP PIP 5-K\textalpha. Cells were grown on glass cover slips over night prior to transfection. 16 hours post-transfection, cells were stimulated with H\textsubscript{2}O\textsubscript{2}. Shown is a representative image of cells prior to stimulation and 60 min post stimulation with H\textsubscript{2}O\textsubscript{2}. (C) UV-irradiation causes delocalisation of PIP 5-K from the plasma membrane. Identical to Figure 6B however 16 hours post-transfection, cells were stimulated with UV. Shown is a representative image of cells prior to stimulation and 5 min after treatment with UV. (D) PtdIns(4,5)P\textsubscript{2} depletion does not cause delocalisation of PIP 5-K from the plasma membrane. Shown are the confocal images of the mid-section of HeLa cells transfected with GFP PIP 5-K, mRFP-PH and truncated NKA receptor. Cells were grown on glass cover slips over night prior to transfection. 16 hours post transfection cells were stimulated with NKA and images taken prior to stimulation and for 30 minutes after stimulation. Shown is an image of a representative cell prior to stimulation and 20 seconds after NKA addition. It should be noted that throughout the entire time course of the experiment, GFP PIP 5-K remained localised at the plasma membrane.
The translocation of mRFP-PH<sup>PLC</sup> in cells treated with H<sub>2</sub>O<sub>2</sub> (data not shown). These data suggested that distinct signalling events are initiated after H<sub>2</sub>O<sub>2</sub> treatment that attenuate PIP 5-K activity at the plasma membrane leading to transient inactivation of the enzyme and displacement of the enzyme away from the plasma membrane. Both these events would serve to deplete PtdIns(4,5)P<sub>2</sub> levels after H<sub>2</sub>O<sub>2</sub> treatment, which we have demonstrated are essential for stress-induced apoptosis.

**Discussion**

We show that apoptotic stress stimuli trigger irreversible PtdIns(4,5)P<sub>2</sub> depletion. PtdIns(4,5)P<sub>2</sub> depletion occurs almost immediately after H<sub>2</sub>O<sub>2</sub> or UV-irradiation treatment and is independent of caspase activation. Therefore we propose that H<sub>2</sub>O<sub>2</sub> or UV-irradiation mediated PtdIns(4,5)P<sub>2</sub> depletion constitutes an early apoptotic signalling event. A previous report (Mejillano, 2001) has shown that activated caspase-3, cleaves and inactivates human PIP 5-K<sub>α</sub> (homologue of the murine PIP 5-K<sub>β</sub>). Primary structure analysis of the other PIP 5-K isoforms (murine PIP 5-K<sub>α,γ</sub> or H<sub>1</sub>), failed to reveal homologous caspase cleavage sites indicating that these enzymes are unlikely to be directly regulated by caspase cleavage. However, our data and the data from Mejillano et al. indicate that apoptotic stimuli down-regulate PIP 5-K activity in both a caspase-dependent and -independent manner. It is possible that caspase-independent mechanisms are required during the early stages of apoptosis and that caspase-dependent mechanisms are employed later on during apoptosis (post-caspase activation) for the inactivation of the murine β isoform (homologue of human PIP 5-K<sub>α</sub>). Furthermore, as isoforms of PIP 5-K appear to have non-redundant functions, caspase-dependent and -independent mechanisms may serve to differentially regulate specific pools of PtdIns(4,5)P<sub>2</sub> (nuclear versus cytoplasmic for example).

Expression of PIP 5-K<sub>α</sub> attenuates both H<sub>2</sub>O<sub>2</sub> and UV-irradiation induced apoptosis and moderately inhibits TNFα/cyclohexaminde-induced apoptosis (JRH, unpublished data), the latter being in good agreement with a previous report (Mejillano, 2001). However, we were unable to observe decreases in PtdIns(4,5)P<sub>2</sub> levels after TNFα/cyclohexamidine treatment suggesting that certain apoptotic stimuli (stress stimuli) are more dependent on PtdIns(4,5)P<sub>2</sub> depletion than others (death receptor agonists such as TNFα). A role for PIP 5-K in death receptor mediated cell death has been previously suggested as RNAi mediated suppression of PIP 5-K<sub>γ</sub> sensitises HeLa cells to apoptosis induced by TRAIL (Aza-Blanc, 2003). The anti-apoptotic effect of PIP 5-K is dependent upon kinase activity as a kinase dead version of PIP 5-K offered negligible protection against H<sub>2</sub>O<sub>2</sub> and UV-mediated cell death. As LY294002 can block PIP 5-K-induced PtdIns(3,4,5)P<sub>3</sub> formation but not its ability to attenuate apoptosis, it is unlikely that PtdIns(3,4,5)P<sub>3</sub> generation is the mechanism for the inhibition of stress induced-apoptosis. In agreement with this data, expression of PIP 5-K failed to augment or affect PKB activation although it completely rescued cells from H<sub>2</sub>O<sub>2</sub>-induced apoptosis. We suggest that PtdIns(4,5)P<sub>2</sub> depletion enables an essential event for apoptosis, such as the inhibition of a survival strategy or the activation of a pro-apoptotic signal. With respect to this issue, previous *in vitro* data suggested that PtdIns(4,5)P<sub>2</sub> can inhibit
caspase 8 and 9 activity. However, so far we were unable to demonstrate that overexpression of PIP 5-Kα, which attenuated H2O2-induced apoptosis, also blocked caspase activation (unpublished data). Modulation of the cytoskeleton using agents such as cytochalasins potently induces apoptosis (Yamazaki 2000; Rubtsova 1998). As PtdIns(4,5)P2 is a key regulator of cytoskeletal dynamics, an enticing possibility is that PtdIns(4,5)P2 depletion induces apoptosis through the modulation of the actin cytoskeleton.

In this paper, we present evidence to support the idea that inactivation of PIP 5-K causes sustained PtdIns(4,5)P2 depletion during H2O2-induced apoptosis. Negative regulation of murine PIP 5-Kα (homologue of human PIP 5-Kβ) occurs through both the inhibition of PIP 5-K catalytic activity and through its relocalisation away from its substrate at the plasma membrane. In our model, inactivation of PIP 5-K by H2O2 prevents re-synthesis of PtdIns(4,5)P2 thereby depleting the cell of this lipid. Interestingly, both H2O2 and UV-irradiation can also activate PLC and PtdIns(4,5)P2 hydrolysis (Scheiven, 1993). Thus, the coordinated activation of PLC and PtdIns(4,5)P2 hydrolysis (Scheiven, 1993). Thus, the coordinated activation of PLC and inactivation of PIP 5-K may be the underlying mechanism for stress-induced PtdIns(4,5)P2 depletion. In S. cerevisiae, mutation of the phospholipase C gene, ptc1, sensitises yeast to UV-irradiation (Andoh, 1998). Furthermore, up-regulation of PKC signalling in mammalian cells can attenuate UV-irradiation induced apoptosis (Matsumura, 2003). Both these data indicate that PLC activity and the resulting activation of PKC may contribute to apoptotic resistance. Thus, maintenance of PtdIns(4,5)P2, by over-expression of PIP 5-K, may attenuate apoptosis through maintenance of the PLC signalling pathway.

PtdIns(4,5)P2 is a key intracellular phosphoinositide required for the regulation of many different signalling pathways including the PLC and PtdIns(3,4,5)P3/PKB pathway. Furthermore PtdIns(4,5)P2 is essential for vesicle generation and actin dynamics. Dysfunction of these processes is unlikely to be compatible with life. PIP 5-K appears to be a central player in response to oxidative damage. For that reason it is tempting to speculate that PIP 5-K acts as an essential intracellular stress sensor, governing the switch between survival and apoptosis. For example at the point where accumulation of oxidative damage abrogates PIP 5-K activity, the decision is made to embark into a pathway of programmed cell death. The ensuing apoptotic signalling cascade may be triggered by the concomitant decreases in PtdIns(4,5)P2 levels. It follows therefore that maintenance of PIP 5-K activity and hence PtdIns(4,5)P2 levels will enable cells to escape apoptosis in response to cellular stress. Clinically, this raises the issue of PIP 5-K in diseases that are characterised by high levels of oxidative stress, such as certain human cancers (Sztarowski, 1991). It is tempting to speculate that these tumour cells may maintain higher than normal levels of PtdIns(4,5)P2 or alternatively that they have developed a PIP 5-K that is insensitive to H2O2. Intriguingly, evidence for aberrant PIP 5-K activity already exists in tumours (Singhal, 1994A; 1994B). In conclusion, PtdIns(4,5)P2 has an essential role in cellular survival and maintaining levels of this lipid is sufficient to circumvent programmed cell death. Accordingly, certain apoptotic stimuli signal the depletion of PtdIns(4,5)P2 in the early stages of apoptosis by inactivating PIP 5-K.

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Materials and Methods

Materials, cell culture and Plasmids

Synthetic PtdIns(4)P dipalmitoyl esters were purchased from Echelon (Salt Lake City, UT). Phosphatidylerine, phosphatidic acid, Z-VAD-fmk and hydrogen peroxide were all purchased from Sigma (St. Louis, MO). Topro-Red was purchased from Molecular Probes. ZVAD-fmk was purchased from Sigma. GFP, YFP and CFP-PH<sub>2LC</sub> constructs are described elsewhere. GFP-PIP<sub>2CL</sub> was a kind gift from Professor P. Downes and was subsequently cloned into a mRFP construct. GFP-Histone H2B and activated G<sub>q</sub>* was provided by Prof. W Moolenaar. GFP-PIP 5-Kα and myc-pcDNA PIP 5-Kα were PCR cloned as previously described in our laboratory from a murine brain cDNA library (Divecha, 2000). The CAAX PI 3-kinase construct was a gift from Dr Len Stephens. HeLa cells were routinely cultured in 10 % fetal bovine serum (Gibco) in dulbecco’s modified Eagle’s medium (DMEM) and transfected using Fugene transfection reagent (Boehringer-Mannheim).

Apoptosis Assays

During our study, two methods were employed to examine cellular apoptosis. The first method was based upon the nuclear morphology of cells visualised using fluorescence microscopy after staining with bisbenzimide (Hoescht 33258, Sigma). Cells were collected 24 hours post-apoptotic stimulation including those that had detached from the cell culture plate. Cells that were still attached were washed twice with PBS (washes were saved) and removed by trypsinization. All fractions including PBS wash steps were then pooled and washed a further two times with PBS. The cellular pellet was then fixed in 50 μl of a 3.7 % (v/v) formaldehyde/PBS solution. After 10 min at room temperature, the fixative was removed and the cells were resuspended in 15 μl of PBS containing 16 μg/ml bisbenzimide. A 10 μl aliquot was placed on a glass slide, and approximately 500 cells per slide were scored in duplicate or triplicate for the incidence of apoptotic nuclear changes under a Zeiss Axiovert 135 fluorescence microscope. To be classified as apoptotic we scored the incidence of fragmented nuclei only and did not include the incidence of condensed or partially condensed nuclei as apoptotic. This generated highly reproducible data sets. The second method was also based on nuclear morphology and was used for imaging experiments. Briefly, cells were transfected with GFP Histone2B. After apoptotic stimulation cells were fixed and treated with the nuclear stain Topro-Red and imaged using confocal microscopy (Leica).

PKB assay

Activation of PKB was determined using a phosphospecific antibody that recognises Serine<sup>473</sup> of PKB or Throneine<sup>308</sup>. The PKB activity assays were performed as described previously (Welch, 1998).

FRET and confocal imaging

For confocal imaging, a Leica DM-IRBE inverted microscope fitted with a TCS-SP scanhead (Leica, Mannheim, Germany) was used. Excitation of enhanced GFP was with the 488-nm argon ion laserline, and emission was collected at 500-565 nm. For translocation studies, series of confocal images were taken at 2-10-s intervals and stored on disc. PtdIns(4,5)P<sub>2</sub> FRET studies were performed as described previously (ref) and PtdIns(3,4,5)P<sub>3</sub> FRET studies adapted from these methods. Visualisation and analysis was performed off-line using LCS and Qwin software (Leica). Qwin software was used to automate the assignment of regions of interest.

[<sup>32</sup>P]-orthophosphate labelling and PIP 5-K in vitro assays

Transfected or non-transfected cells were orthophosphate labelled as previously described. After extraction, phospholipids were treated with monomethylamine and the deacylated products analysed using PEI-cellulose plates (Whatman) as described previously (Halstead, 2001). For the in vitro PIP 5-K assays, cells were transfected as described and left for 16 h, after which they were lysed [1 ml lysis buffer (50 mM Tris pH 8.0, 100 mM NaCl, 1% NP-40)], scraped, and nuclei and cellular debris removed by centrifugation (14 000 r.p.m., 4°C Eppendorf centrifuge). Immunoprecipitation of the endogenous PIP 5-K from Hek293T cells was carried out using a polyclonal antibody raised against a peptide present all three isoforms of PIP 5-K. The immunoprecipitates were collected using protein G–Sepharose, and washed three times with IP wash buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA 0.1% Tween-20), then twice with 1x PIP 5-K buffer. For the PIP 5-K assays, lipid vesicles were
prepared using 1 nmol of PtdIns4P together with 1 nmol PtdSer and 3 nmol of PtdOH. Reactions were carried out at 30°C for 20 min in 1 x PIP kinase
buffer (50 mM Tris pH 7.4, 10 mM MgCl2, 1 mM EGTA, 70 mM KCl) containing cold ATP (20 µM) and 1 µCi of [32P]ATP in a final volume of 100 µl. Reactions were quenched with 0.5 ml of chloroform:methanol [1:1 (v:v)] and the phases were split by the addition of 125 µl of 2.4 M HCl. The lower phases were removed to a new tube, dried and separated by TLC either using an alkaline solvent [chloroform:methanol:ammonia (28%):water 45:35:2:8]. Incorporation into PtdIns(4,5)P2 was quantitated using a phosphoimager.

References


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