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The role of inositol signaling in the control of apoptosis

Philip W. Majerus*, Jun Zou, Jasna Marjanovic, Marina V. Kisseleva, and Monita P. Wilson
Division of Hematology Washington University School of Medicine, 660 South Euclid St. Louis, Mo. 63110

Introduction

Intracellular signaling reactions are frequently carried out by changes in phosphorylation. There are three main systems of phosphorylation as shown in figure 1.

The oldest and most simple is the cAMP/cGMP system which is conserved from bacteria to humans and is a relatively simple on/off switch. Next is the inositol phosphorylation system which does not exist in bacteria but is found in all eukaryotes. This system is more complex but manageable with 63 possible soluble inositol phosphates, over half of which have been found in cells to date. In addition there are 8 known inositol lipids as described in figure 2. The parent lipid is phosphatidylinositol which is esterified to diacylglycerol in the one position leaving 5 potential hydroxyl groups for further phosphorylation. Only the 3, 4, and 5 positions have been found to be phosphorylated in cells and inositol lipids with every possible combination of these phosphates have been identified in cells as shown in figure 3, depicting the current status of inositol signaling in humans. Initially it was thought that the phosphoinositides were simply precursors of the water soluble inositol phosphate messengers but it is now clear that the lipids themselves have diverse signaling functions (York, 2006; Gonzales and Anderson, 2006). In this report we will describe studies showing that both inositol phosphates and phosphoinositides are modulators of apoptosis. We recently defined the pathway for synthesis of inositol hexakisphosphate (InsP₆) in mammalian cells as shown in figure 4 (Verbsky *et al.*, 2005). *Role of InsP₆ in apoptosis*

The precursor inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) is formed by hydrolysis of PtdIns(4,5)P₂ by one of the many phospholipase C enzymes. According to J. York (York, 2006) this reaction evolved for production of the higher inositol phosphates rather than to evoke calcium mobilization since fungi do not have IP₃ receptors but do require InsP₆ and other highly phosphorylated inositols for survival (York, 2006). In mammals, Ins(1,4,5)P₃ is converted to inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) by inositol trisphosphate 3-kinase (E.C. 2.7.1.127) and further metabolized to inositol 1,3,4-trisphosphate (Ins(1,3,4)P₃) by inositolpolyphosphate 5-phosphatase (E.C. 3.1.1.56). Ins(1,3,4)P₃ is then converted to inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P₄) by inositol 1,3,4-trisphosphate 5/6-kinase (5/6-kinase) (E.C.2.7.1.159). This rather unique enzyme is able to phosphorylate its substrate in either the 5 or 6 position of the inositol ring although in cells the net effect is the formation of the tetrakisphosphate with the 6-position phosphorylated since any 5 phosphate containing product is rapidly converted back to Ins(1,3,4)P₃ by the above pathway (Miller *et al.*, 2005). 5/6-kinase is at a critical branch point of inositol phosphate metabolism which leads to the

*Corresponding author. PHIL@dom.wustl.edu.

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formation of all of the highly phosphorylated inositol phosphates including InsP_6 and as noted below is the rate limiting step in InsP_6 synthesis (Verbsky *et al.*, 2005). $\text{Ins}(1,3,4,6)\text{P}_4$ is then converted to inositol 1,3,4,5,6-pentakisphosphate (InsP_5) by inositol phosphate multikinase (E.C. 2.7.1.151). InsP_5 is then converted to InsP_6 by InsP_5 -2-kinase (E.C. 2.7.1.155).

That 5/6-kinase is the regulated step in InsP_6 synthesis is indicated by several lines of evidence: 1) overexpression of 5/6-kinase in cells increases the level of InsP_6 greatly while RNAi of the enzyme reduces InsP_6 levels in cells (Verbsky *et al.*, 2005). 2) The enzyme is inhibited by many of the end products of highly phosphorylated inositols as shown in table 1.

The most potent inhibitor of the enzyme is inositol 3,4,5,6-tetrakisphosphate which has a K_i several orders of magnitude greater than the others. This inositol phosphate is also a substrate for the enzyme (Yang *et al.*, 2000). The inhibition constants shown in table 1 are similar to previously reported inhibition constants obtained using partially purified enzyme (Hughes *et al.*, 1994). 3) Overexpression or RNAi of inositol phosphate multikinase that is downstream of 5/6-kinase has no effect on cellular levels of InsP_5 or InsP_6 (Verbsky *et al.*, 2005). 4) Overexpression of InsP_5 2-kinase only increases InsP_6 levels to the extent of converting any InsP_5 to InsP_6 (Verbsky *et al.*, 2005). 5) A mutation in a maize plant with low InsP_6 levels was identified by positional cloning and it was found that the mutation was in the *Zea mays* 5/6-kinase gene (Shi *et al.*, 2003).

When 5/6-kinase is transiently transfected into HEK 293 cells which have been labeled with [^{32}P]-inorganic phosphate, immunoprecipitation of 5/6-kinase yields a phosphorylated enzyme (Sun *et al.*, 2002). Phospho-amino acid analysis reveals that 5/6-kinase is phosphorylated on serine and tyrosine residues (fig. 5a). However, when recombinant 5/6-kinase is heterologously expressed in and purified from *E. coli*, and used in an *in vitro* phosphorylation assay, phosphoamino acid analysis of the resulting phosphorylated 5/6-kinase does not yield phosphorylated serine and tyrosine residues. As shown in figure 5a, digestion of autophosphorylated 5/6-kinase yields only inorganic phosphate. To further explore the nature of the phosphorylation, the chemical stability of autophosphorylated 5/6-kinase was assessed using the method described by Muimo *et al.* (Muimo *et al.*, 2000). As shown in figure 5b, the phosphate bond is acid labile, base stable, and somewhat labile in the presence of neutral hydroxylamine. This pattern of stability is consistent with the bond being a phosphoramidate, since acylphosphates are both acid and base labile, whereas phosphoserine and threonine residues are stable under both conditions.

The nature of the amino acid residue phosphorylated by the enzyme is uncertain but seems most likely to be a histidine based on the above properties. This labile bond was also seen with immunoprecipitated endogenous enzyme as seen from the finding of inorganic phosphate after acid hydrolysis of the enzyme as shown in figure 5a.

5/6-kinase also has protein kinase activity phosphorylating proteins involved in the cellular response to stress including c-Jun, Ikb α , ATF2 and p53 (Wilson *et al.*, 2001). This suggested to us that 5/6-kinase might play a role in the apoptotic response to cellular stress. Further support for this suggestion came from the fact that 5/6-kinase from *E. histolytica* was cloned as a protein induced by cellular stress (Field *et al.*, 2000).

We examined the role of 5/6-kinase in apoptosis induced by tumor necrosis factor α or FAS by both overexpressing the enzyme in HEK 293 cells or by RNAi of the endogenous enzyme in these cells (Sun *et al.*, 2003). We found that overexpression of 5/6-kinase protects cells from apoptosis as shown in figure 6 while RNAi enhances it (data not shown). We found however that these effects were not mediated by the protein kinase activity of the enzyme but rather its ability to phosphorylate its inositol phosphate substrate. We found that overexpression of InsP_5 2-kinase, the final enzyme in the pathway to InsP_6 , also protects cells from apoptosis

and RNAi of this enzyme enhances apoptosis indicating that the effects noted emanated from either the altered levels of InsP_6 or one of the inositol pyrophosphates formed from InsP_6 (Verbsky and Majerus, 2005). We have also found that InsP_6 is essential for life in mammals since deletion of either InsP_5 2-kinase or inositol phosphate multikinase in mice has an embryonic lethal phenotype (Verbsky *et al.*, 2005b, Frederick *et al.*, 2005).

Role of Phosphatidylinositol 5-phosphate in apoptosis

Phosphatidylinositol 5-phosphate (PtdIns5P) is the most recently discovered inositol lipid and its function is least understood (Rameh *et al.*, 1997). We now describe studies showing that this lipid plays a critical role in controlling p53-dependent apoptosis (Zou *et al.*, 2007). PtdIns5P is formed in mammalian cells by the action of PtdIns(4,5) P_2 4-phosphatase (no E.C. number as yet) (type I 4-phosphatase) (Ungewickell *et al.*, 2005) as shown in figure 3. Thus PtdIns(4,5) P_2 is metabolized to both PtdIns4P and PtdIns5P.

We have also shown that PtdIns5P is a substrate for phospholipase $\text{C}\gamma 1$ (E.C. 3.1.4.11) forming inositol 1,5-bisphosphate ($\text{Ins}(1,5)\text{P}_2$) as its product (unpublished). PtdIns5P is a good substrate for the enzyme with K_m (120 μM) and V_m (15 $\mu\text{moles/min/mg}$ protein) similar to those found using PtdIns(4,5) P_2 as substrate (data not shown). Furthermore the $\text{Ins}(1,5)\text{P}_2$ product is further metabolized by inositol polyphosphate 1-phosphatase (E.C. 3.1.3.57) to form inositol 5-phosphate. This gives rise to two previously unknown inositol phosphates whose functions in cell signaling remain to be discovered.

A role for PtdIns5P in p53-dependent apoptosis has been suggested from previous studies. Gozani and co-workers showed that the nuclear factor ING2 was required for efficient acetylation of p53 (Gozani *et al.*, 2003). Acetylation of p53 stabilizes the molecule preventing its proteosomal degradation thereby enhancing apoptosis. RNAi of ING2 reduced apoptosis. Transfection of cells with phosphatidylinositol phosphate kinase (PIPK) type II β (E.C. 2.7.1.149) which converts PtdIns5P to PtdIns(4,5) P_2 also decreases apoptosis leading the authors to conclude that PtdIns5P formed a complex with ING2 which acted as a cofactor for p53 acetylation, although PtdIns5P levels were not measured in that study. This work was done prior to the discovery of type I 4-phosphatase and thus the source of PtdIns5P was not known. We have now shown that tetracycline-inducible stable HEK 293 cells overexpressing type I 4-phosphatase have a 20% reduction in PtdIns(4,5) P_2 levels and have a 50% increase in PtdIns5P (Ungewickell *et al.*, 2005; Zou *et al.*, 2007). When these cells are subjected to genotoxic stress using either etoposide or doxorubicin, p53-dependent apoptosis is increased. Induction of type I 4-phosphatase expression with tetracycline in these cells increases PtdIns5P levels and promotes p53 stability thereby increasing apoptosis. Conversely RNAi of the enzyme decreases p53 stability and inhibits apoptosis. We measured the half life of p53 in control cells and in cells treated with an RNAi construct against type I 4-phosphatase by inhibiting protein synthesis with cycloheximide and Western blotting for p53 at various times thereafter. In control cells the half life of p53 was 7 hours while in RNAi treated cells it was reduced to 1.8 hours. The ability of type I 4-phosphatase to stabilize p53 was ING2 dependent since the effect was lost upon RNAi of ING2. The results are consistent with the idea that PtdIns5P forms a complex with ING2 and both are required for acetylation and stabilization of p53. We measured acetylation of p53 directly using antibodies specific for acetylated p53 to confirm the above hypothesis. Type I 4-phosphatase is primarily cytosolic where it is found bound to the surface of late endosomal/lysosomal membranes (Ungewickell *et al.*, 2005). Since ING2 and p53 are nuclear proteins we investigated the possibility that cellular stress caused a redistribution of type I 4-phosphatase to the nucleus. HeLa cells were treated with 100 μM etoposide for 4 hours and cells were harvested and nuclei were isolated. We found that prior to etoposide treatment 10% of cellular type I 4-phosphatase was nuclear which increased to 40% of the total after etoposide. The total level of type I 4-phosphatase in cells was unchanged so this represents a

migration of the enzyme into the nucleus. This nuclear import effected a 50% increase in nuclear PtdIns5P. Our interpretation of these results is summarized in figure 7.

Within the nucleus PtdIns5P levels are determined by the opposing actions of type I 4-phosphatase which forms PtdIns5P and that of PIPK type II β which converts it to PtdIns(4,5)P₂. Upon genotoxic stress type I 4-phosphatase migrates to the nucleus where it effects an increase in PtdIns5P which complexes with ING2 to promote acetylation of p53, stabilizing it and thereby increasing apoptosis. It is not clear how type I 4-phosphatase is able to get into the nucleus. It does not contain any canonical nuclear localization sequences and it does contain putative membrane spanning regions (one or two). Perhaps it gains access to the nucleus by some type of vesicle trafficking wherein a vesicle fuses with the nuclear membrane. Future experiments will be directed at resolving these questions.

Summary

Inositol signaling reactions are very broad in scope affecting many cellular functions. In this report we describe experiments showing that two distinct parts of this system play pivotal roles in an important cellular event, namely apoptosis. Apoptosis is important for organ development and also for controlling cell survival after various stresses which include DNA damage and other proapoptotic stimuli such as tumor necrosis factor α . We show that the inositol phosphate InsP₆ or one of its pyrophosphate metabolites determines the extent of apoptosis following tumor necrosis factor α treatment whereby increased cellular levels of InsP₆ protect from apoptosis and decreased levels promote it. Cellular levels of InsP₆ are determined by the activity of 5/6-kinase since this is the rate limiting enzyme in production of the highly phosphorylated inositol phosphates including InsP₆. A lipid inositol metabolite PtdIns5P is also critical in regulating the activity of p53-dependent apoptosis. This phospholipid is formed in cells by the action of type I 4-phosphatase on PtdIns(4,5)P₂. PtdIns5P stabilizes p53 by promoting its acetylation in complex with the nuclear factor ING2. Upon genotoxic stress type I 4-phosphatase migrates to the nucleus where it catalyzes the formation of PtdIns5P to stabilize p53 and increase apoptosis.

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Intracellular signaling is controlled by changes in phosphorylation: three main systems

1. cAMP/cGMP system (ie. ATP-cAMP-AMP)
 - cyclases and phosphodiesterases
2. Inositol phosphorylation
 - 63 Soluble inositol phosphates
 - 8 Inositol lipids
 - kinases and phosphatases
3. Protein phosphorylation on T/S/Y
 - kinases and phosphatases

System 1: simple switch

System 2: complex but manageable

System 3: infinite complexity

Fig. 1.
Systems of phosphorylation in cells.

Phosphatidylinositol Signaling

- Phosphatidylinositol is esterified in the 1 position leaving 5 potential sites for phosphorylation
- To date only the 3, 4, and 5 positions have been found to be phosphorylated
- Every combination of phosphorylation at the 3, 4, and 5 positions have been found in cells; all have functions

ie. PI(3)P, PI(4)P, PI(5)P, PI(3,4)P₂, PI(3,5)P₂, PI(4,5)P₂, and PI(3,4,5)P₃

Fig. 2.
Phosphorylation of inositol lipids.

Fig. 3.
Cellular phosphoinositide metabolism.

Fig. 4.
The pathway for InsP₆ synthesis in mammalian cells.

Fig. 5. Phosphorylation of 5/6-kinase. A. Phosphoamino acid analysis of phosphorylated 5/6-kinase immunoprecipitated from HEK 293 cells labeled with [^{32}P]-inorganic phosphate (left panel) and that of purified 5/6-kinase incubated in an *in vitro* autophosphorylation reaction (right panel) under previously described conditions (Wilson *et al.*, 2001). B. Chemical stability of autophosphorylated 5/6-kinase. Purified 5/6-kinase was allowed to autophosphorylate in the presence of [γ - ^{32}P]-ATP, incubated under various conditions for 10 min. at 30°C, run on SDS-PAGE, transferred to a PVDF membrane and autoradiographed. Incubation conditions include reaction with 20 mM Tris-HCl, pH 7.6 as a control (lane 1), reaction with 0.1 N HCl, pH 1.0 (lane 3), reaction with 0.1 N NaOH, pH 13 (lane 4), and 0.8 M hydroxylamine with 0.1 M acetate, pH 5.2 (lane 6). Lanes 2 and 5 are empty.

Fig. 6. Apoptosis induced by TNF α in HEK 293 cells expressing 5/6-kinase or vector alone. Cells were treated with TNF α (1 ng/ml) and CHX (0.5 μ g/ml) for 6 hours, then stained with APOPercentage (Biocolor, Belfast, Northern Ireland) as per manufacturer's instructions.

Fig. 7.
Model depicting the PtdIns-5-P stress response mediated by type I 4-phosphatase.

Table 1

Inhibition of calf brain 5/6-kinase by inositol polyphosphates

Inositol polyphosphate	K _i
InsP ₆	37 μM
InsP ₅	2.5 μM
Ins(3,4,5,6)P ₄	30 nM
Ins(1,3,4,5)P ₄	3.5 μM
Ins(1,3,4,6)P ₄	3.5 μM
Ins(1,2,5,6)P ₄	52 μM
Ins(1,4,5)P ₃	31 μM
Ins(1,5,6)P ₃	2 μM
Ins(2,4,5)P ₃	15 μM
Ins(2,4)P ₂	290 μM
Ins(1,4)P ₂	95 μM
Ins(4,5)P ₂	225 μM

The K_i was determined using the following equation: $v = V_m[S]/K_m(1 + [I]/K_i) + S$