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Expression of inositol 1,3,4-trisphosphate 5/6-kinase (ITPK1) and its role in neural tube defects

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Introduction

Inositol can be obtained through the diet or synthesized by some cells from glucose-6-phosphate (Daughaday *et al.*, 1955; Holub, 1986). Through the actions of kinases and phosphatases, inositol can be phosphorylated and dephosphorylated to form a large number of intermediates in a complex metabolic pathway. With six positions available for phosphorylation there are roughly 70 potential intermediates, including both the lipid phosphatidylinositols and the soluble forms of inositol, several of which can contain pyrophosphate bonds (Irvine *et al.*, 2001).

Inositol, 1,3,4-trisphosphate 5/6-kinase (ITPK1, E.C. 2.7.1.159) plays a pivotal role in inositol metabolism, is conserved from plants to humans, and is even found in *Entamoeba histolytica* (Field *et al.*, 2000; Wilson *et al.*, 1996; Wilson *et al.*, 1997). The known phosphoryl transferase activities of ITPK1 are shown diagrammatically in Fig. 1. The substrate inositol 1,3,4-trisphosphate (Ins(1,3,4)P₃) sits at a major branch point in the inositol metabolic pathway. It can be phosphorylated by ITPK1 in either the D5 or the D6 position of the inositol ring to form inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) or inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P₄), respectively (Fig. 1A). Either tetrakisphosphate (IP₄) product can also serve as a substrate in an isomerization reaction (Ho *et al.*, 2002; Miller *et al.*, 2005). In addition, ITPK1 has also been shown to reversibly phosphorylate another IP₄ isomer, inositol 3,4,5,6-tetrakisphosphate (Ins(3,4,5,6)P₄), to form inositol pentakisphosphate (IP₅) (Fig. 1B) (Chamberlain *et al.*, 2007; Ho *et al.*, 2002; Miller *et al.*, 2005; Yang *et al.*, 2000).

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The formation of Ins(1,3,4,6)P₄ by ITPK1 represents the rate-limiting step in the formation of the higher phosphorylated forms of inositol in mammalian cells (Verbsky *et al.*, 2005) including IP₅, IP₆, and the pyrophosphates diphosphoinositol-pentakisphosphate (IP₇) and bisdiphosphoinositol tetrakisphosphate (IP₈), shown diagrammatically in Fig. 2.

Functions of IP₆

In recent years, a number of functions for the more highly phosphorylated forms of inositol have come to light. IP₆ has been shown to be an integral component of three proteins: adenosine deaminase (E.C.3.5.4.4) (Macbeth *et al.*, 2005), arrestin (Milano *et al.*, 2006), and the receptor for the plant hormone auxin (Tan *et al.*, 2007). In addition to these structural roles, IP₆ has been implicated in a wide range of cellular processes, including mRNA export from the nucleus (Alcazar-Roman *et al.*, 2006; Feng *et al.*, 2001; Weirich *et al.*, 2006; York *et al.*, 1999), regulation of transcription (Odom *et al.*, 2000), increasing the ATPase activity of the dead box protein Dbp5 (E.C.3.6.1.-) (Alcazar-Roman *et al.*, 2006), binding to the clathrin assembly proteins AP2 and AP180, inhibiting clathrin cage assembly *in vitro* (Chadwick *et al.*, 1992; Norris *et al.*, 1995; Theibert *et al.*, 1991; Ye *et al.*, 1995), inhibiting serine and threonine protein kinases (Larsson *et al.*, 1997), stimulating protein kinases including casein kinase-2 (E.C. 2.7.11.1) (Hilton *et al.*, 2001; Solyakov *et al.*, 2004), regulating nonhomologous DNA end joining of double strand breaks (Hanakahi *et al.*, 2000; Hanakahi *et al.*, 2002), and endocytosis (Hoy *et al.*, 2002). Most recently, IP₆ has been shown to activate the cysteine protease domains of both the *Vibrio cholera* RTX (repeats in toxin) (Lupardus *et al.*, 2008; Prochazkova *et al.*, 2008) and the *Clostridium difficile* A and B toxins (Pruitt *et al.*, 2009).

To underscore the importance of IP₆ in cells, deletion of the murine genes encoding the two other kinases leading to the synthesis of IP₆ results in early embryonic lethality, suggesting that IP₆ is essential for life (Frederick *et al.*, 2005; Verbsky *et al.*, 2005; Wilson *et al.*, 2009).

Results and Discussion

Generation of an ITPK1-deficient mouse

We have generated a genetically engineered mouse harboring a hypomorphic allele in *Itpk1* (Wilson *et al.*, 2009). The β -galactosidase (β gal) containing gene trap cassette is inserted in the second intron of the *Itpk1* gene, and by alternative splicing produces both an *Itpk1*- β gal fusion transcript as well as a wild type, full-length transcript. RT-PCR using mRNA extracted from brain tissue of wild type animals as well as those heterozygous and homozygous for the gene trap allele show that all animals produce mRNA for *Itpk1* (Fig. 3). RT-PCR of L19, a ribosomal protein, is shown as a loading control. The levels of *Itpk1* mRNA vary in both the heterozygotes and homozygotes, consistent with varying levels of ITPK1 protein produced by these animals (Wilson *et al.*, 2009).

Expression of ITPK1 in mouse tissues

We have previously reported that *Itpk1* is widely expressed throughout the nervous system in developing embryos and in adult mice (Wilson *et al.*, 2009). Using Xgal staining, we have also analyzed *Itpk1* expression in other mouse tissues. Expression was seen in the vascular smooth muscle cells in arterioles of kidney (Fig. 4A-C), thorax (Fig. 4D), and gut (Fig. 4E-F). In the digestive tract, expression is seen in serosal cells (Fig. 4E), visceral smooth muscle cells (Fig. 4F-H), and crypt cells (Fig. 4I). Expression was also evident in the smooth muscle cells in the vas deferens (Fig. 4J), neuroepithelial derivatives including brainstem neurons (Fig. 4K), and pia mater (Fig. 4L).

Neural tube defects in *Itpk1* hypomorphic embryos

Between embryonic day (E) 9.5 and E11.5, 12% of embryos resulting from mating animals homozygous for the gene trap allele have some form of neural tube defect (NTD), either spina bifida, exencephaly, or both (Wilson *et al.*, 2009). The severity of the NTDs is quite variable, ranging from the mild caudal defects (Fig 5D), to moderately severe cranial defects (Fig. 5A-B) to cases of exencephaly that would be incompatible with life (Fig. 5C). The animals used in these experiments are of a mixed genetic background (C57Bl/6 $\times$ 129(P2)Ola). Animals are currently being backcrossed to pure genetic backgrounds representing each of the parental strains to determine whether genetic background plays a role in the incidence of NTDs, as has been shown to be the case for NTDs in the *curly tail* mouse, a mouse model for folic acid-resistant NTDs (Letts *et al.*, 1995; Neumann *et al.*, 1994; Van Straaten *et al.*, 2001). Defects in neural tube development have also been shown to occur in mice harboring deletion of PIP5KI γ (Wang *et al.*, 2007) further establishing the role of inositol signaling in neuronal development.

Measurements of myo-inositol

The NTDs observed in the *Itpk1* hypomorphs likely represent an example of folate-resistant NTDs. In humans, folic acid supplementation provides a 70% protective effect (Wald, 1991) leaving 30% which are folate-resistant. There are several animal models for folate-resistant NTDs. In some of these, the level of inositol affects the prevalence of the NTDs (Cockroft, 1988; Cockroft *et al.*, 1992; Copp *et al.*, 2003; Reece *et al.*, 1997). In order to determine whether the NTDs seen in the *Itpk1* hypomorphs result from a perturbation in inositol metabolism, animals will be fed diets containing varying levels of inositol and the prevalence of NTDs will be examined. To determine the level of inositol in the mouse chow as well as in the mice, to confirm that the levels of inositol are being altered, it is necessary to be able to assay for inositol concentrations. The enzyme inositol dehydrogenase (E.C.1.1.1.18) converts myo-inositol to *scyllo*-inosose in an NAD⁺-dependent oxidation reaction (Ramaley *et al.*, 1979; Weissbach, 1965). Using purified solutions of inositol in a room temperature reaction containing 50 μ g/ml of inositol dehydrogenase (Sigma Aldrich), the generation of NADH is measured by following the absorbance at 340 nm (Fig. 6A). Reactions reach their maximum absorbance by 70 minutes, as the A₃₄₀ does not increase at time points beyond this time. To ensure that reactions go to completion, the A₃₄₀ is read after 120 minutes. The assay is linear up to 0.25 mM inositol (Fig. 6B).

For determination of inositol content in food (Harlan Teklad), samples were homogenized and extracted as described (Kawa *et al.*, 2003). In addition to the standard mouse chow used by the Washington University Division of Comparative Medicine, two diet samples were obtained from Harlan Teklad (Madison, WI) for assay. Both test diets were based on the AIN-93 nutritionally complete rodent diet (Reeves *et al.*, 1993). The level of inositol in the standard diet was 0.8 mg inositol/kg chow. A diet containing soybean oil (70 g/kg; TD.06706) and a diet containing 7% corn oil (TD.09146) both had reduced levels of inositol: 0.095 mg/kg and 0.072 mg/kg, respectively. We are currently assaying other test diets in order to obtain a relatively inositol free diet.

Summary

ITPK1 is the rate limiting enzyme in the pathway leading to formation of the highly phosphorylated inositol phosphates including IP₆ and the inositol pyrophosphates. One or more of these metabolites are essential for life as deletion of either of the kinases that form IP₅ or IP₆ in mice results in embryonic lethality. We have produced mice harboring a hypomorphic allele for *Itpk1*, and mice homozygous for this gene trap allele produce low but detectable levels of active enzyme. We have studied the expression of *Itpk1* in various tissues and found that

the enzyme is highly expressed in smooth muscle of vessels and other tissues. In addition, these mice have neural tube defects in 12% of homozygous embryos. Since the levels of enzyme expression vary greatly in homozygous animals, we speculate that relative deficiency of one or more inositol phosphates accounts for these defects. We plan to feed an inositol deficient diet or one with supplemental inositol to animals to demonstrate altered prevalence of neural tube defects.

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Fig. 1. Activities of ITPK1. (A) Phosphorylation of Ins(1,3,4)P₃ and isomerization of IP₄ isomers and (B) reversible phosphorylation of Ins(3,4,5,6)P₄.

Fig. 2.
The pathway for the synthesis of the higher phosphorylated forms of inositol in mammalian cells.

Fig. 3. RT-PCR of *Itpk1* (upper panel) and L19 (lower panel) from 2 animals each heterozygous for the gene trap allele (A,B), homozygous for the gene trap allele (C,D), or wild type (E,F). The ribosomal protein L19 is used as a loading control.

Fig. 4.

Expression of *Itpk1* in mouse tissues. Tissue sections from mice containing the *Itpk1*- β gal gene trap allele were subjected to Xgal staining. (A) Whole mount of bisected kidney showing expression in vasculature within the renal cortex (c) but not medulla (me). (B,C) Expression in vascular smooth muscle of renal arterioles (a), including those adjacent to glomeruli (g). (D) Expression in an intercostal arteriole (a) between a rib (r) and skeletal muscle (sk). (E) Expression in smooth muscle cells of the vasa vasorum. (F) Expression in arterioles (a), serosal cells (s) and visceral smooth muscle (m) of the small intestine. (G,H) Expression in visceral smooth muscle (m) of the esophagus (e) and small intestine (si). (I) Weak expression in duodenal crypts. (J) Whole mount showing expression in vas deferens (vd) but not testis (t). (K) Expression in brain stem neurons (n). (L) Expression in pia mater (pm) of the brain.

Fig. 5. NTDs in *Itpk1* hypomorphic mouse embryos. (A) Exencephaly in an E12.5 *Itpk1* hypomorph. The inset shows an H&E stained section through the open NTD (*). (B) Exencephaly in an E12.5 *Itpk1* hypomorph. The inset shows a dorsal view of the open NTD (arrowhead). (C) Severe exencephaly (arrowheads) in an E11.5 *Itpk1* hypomorph. (D) Splayed hindlimbs (hl) in conjunction with a caudal NTD in an E12.5 *Itpk1* hypomorph. The inset shows a higher magnification of the boxed region. Note the open and kinked neural tube (arrowheads).

Fig. 6. Measurement of *myo*-inositol. (A). Time course for inositol dehydrogenase reaction. (B). Concentration-dependence of inositol dehydrogenase.