Cytoophidium Assembly Reflects Upregulation of IMPDH Activity

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ABSTRACT

Cytidine triphosphate synthase (CTPS) and inosine monophosphate dehydrogenase (IMPDH) can form fiber-like subcellular structures termed “cytoophidia” under certain circumstances in mammalian cells. Although it has been shown that filamentation of CTPS downregulates its activity by disturbing conformational changes, the activity of IMPDH within cytoophidia is still unclear. Most previous IMPDH cytoophidium studies were done under conditions involving inhibitors that impair GTP synthesis. Here we show that IMPDH forms cytoophidia without inhibition of GTP synthesis. Firstly, we find that an elevated intracellular CTP concentration or treatment with 3’-deazauridine, a CTPS inhibitor, promotes IMPDH cytoophidium formation and increases the intracellular GTP pool size. Moreover, restriction of cell growth triggers the disassembly of IMPDH cytoophidia, implying that their presence is correlated with active cell metabolism. Finally, we show that IMPDH forms cytoophidia in mouse pancreatic islet cells may respond to nutrient uptake of the animal. Collectively, our findings reveal that formation of IMPDH cytoophidia reflects upregulation of purine nucleotide synthesis, suggesting that the IMPDH cytoophidium plays a role distinct from that of the CTPS cytoophidium in controlling intracellular nucleotide homeostasis.
INTRODUCTION

Nucleotides are not only essential for DNA and RNA synthesis, but are also involved in various metabolic processes and signaling transductions. Inosine monophosphate dehydrogenase (IMPDH) and cytidine triphosphate synthase (CTPS) catalyze the rate-limiting steps of de novo GTP and CTP synthesis, respectively (Lieberman, 1956; Yoshioka et al., 1992).

Recently, several studies have demonstrated that CTPS and IMPDH are involved in the formation of a subcellular fiber-like structure termed “rods and rings” or the “cytoophidium” (Carcamo et al., 2011; Ingerson-Mahar et al., 2010; Ji et al., 2006; Liu, 2010; Noree et al., 2014; Noree et al., 2010). This structure is not membrane-bound and is not associated with any known organelle in mammalian cells (Thomas et al., 2012). Lately, CTPS-based, IMPDH-based and mixed cytoophidia have been found in mammalian cells. Different type cytoophidia displayed similar filamentous morphology, but the proportions of them changed with various inductions. As described, IMPDH inhibitors, mycophenolic acid (MPA) and Ribavirin, induce only IMPDH-based cytoophidia, while 6-diazo-5-oxo-L-norleucine (DON), which interrupts purine and pyrimidine biosynthesis, triggers filamentation of both enzymes (Keppeke et al., 2015). These results suggest that formation of CTPS and IMPDH cytoophidia could be regulated independently.
Multiple inhibitors of nucleotide synthesis have been applied in studying characteristics of IMPDH cytoophidia. Overexpression of fluorescent fusion protein approaches have been adopted in some studies as well (Carcamo et al., 2011; Ji et al., 2006; Thomas et al., 2012). However, information about the dynamics of non-inhibitory treatment that induces IMPDH cytoophidium is required to discover more about their physiological function and regulation.

Herein, we aimed to investigate the regulation of the IMPDH cytoophidium and its putative role in mammalian cell metabolism. We show that regulation of the IMPDH cytoophidium is different from that of the CTPS cytoophidium. Overproduction of CTP or inhibition of CTP synthesis stimulated IMPDH cytoophidium assembly along with an increase of intracellular GTP. We also found that IMPDH cytoophidia spontaneously form in mouse BNL CL2 cells. Maintenance of these cytoophidia is correlated with active cell proliferation. Finally, we show the presence of IMPDH cytoophidia in mouse pancreatic islet cells may correlate with nutrient uptake of the animal. Our findings indicate that IMPDH tends to form cytoophidia when de novo purine synthesis is positively regulated. They also provide new insights into nucleotide metabolism and provide a basis for further research into the potential of the IMPDH cytoophidium as a biomarker or drug target in clinical applications.
MATERIALS AND METHODS

Cell culture

HEK 293T cells, HeLa cells and mouse BNL CL2 cells were cultured in DMEM with high glucose (11965, Gibco, USA) supplemented with 10% fetal bovine serum (04-001, Biological Industries) and 1% penicillin-streptomycin (15140, Gibco). Cells were kept in a 37°C incubator with 5% CO₂. DON (D2141, Sigma-Aldrich) and cytidine were dissolved in water. Mycophenolic acid (M3536, Sigma-Aldrich), guanosine, 3’-deazauridine (sc-394445, Santa Cruz), LY294002 (9901S, Cell Signaling) and rapamycin (R0395, Sigma-Aldrich) were dissolved in DMSO (D2650, Sigma-Aldrich). Chemicals were added in culture medium as described.

Tissue preparation

Animal maintenance and the procedures described herein were approved by the Institutional Animal Care and Use Committee of National Taiwan University. Tissues for paraffin embedding were collected from 20-week-old male ICR mice and then fixed with 4% paraformaldehyde (43368, Alfa Aesar) overnight before proceeding of standard embedding protocol. For wholemount staining, pancreatic islets were isolated from 20-week-old male ICR mice with the method described (Neuman et al., 2014).
**Immunofluorescence**

Cells were cultured on glass cover slides and fixed with 4% paraformaldehyde in PBS for 10 minutes. Fixed cells, mouse islets and tissue sections were incubated in primary antibody in PBS with 2% bovine serum albumin (A9647, Sigma-Aldrich) and 0.2% Triton-X100 (X100, Sigma-Aldrich) for at least 2 hours at room temperature. After washing with PBS, samples were incubated in secondary antibody, which is diluted in the same solution as used in primary antibody dilution. At least 2 hours after secondary antibody reaction, samples were washed and mounted with PBS. Antibodies used in this study included: rabbit anti-human CTPS1 IgG (1:500, GTX105265, GeneTex), mouse anti-human IMPDH1 IgG (1:500, ab55297, abcam), rabbit anti-insulin (1:500, 4590, Cell Signaling).

**Microscopy**

Images were acquired under the 63x objective of a laser-scanning confocal microscope (Leica TCS SP5 II confocal microscope).

**Immunoblotting**

Cell lysates were prepared with RIPA lysis buffer (20-188, Millipore) and were run on a 12% polyacrylamide gel. PVDF membrane (GE Healthcare) was used for protein transfer and the signals of secondary antibodies were detected with X-ray film. Antibodies used for western blot included: rabbit anti-human CTPS1 IgG (1:3000, GTX105265, GeneTex), mouse anti-human IMPDH1 IgG (1:1000, ab55297, abcam), rabbit-anti Akt (1:1000, 4691S, Cell Signaling), rabbit anti-pS473 Akt (1:1000, 4060S,
Cell Signaling) and mouse anti-β-actin (1:10000, A5441, Sigma-Aldrich).

**Nucleotide analysis**

Quantification of intracellular nucleotides was performed following the method in Aughey et al., 2014. In brief, 7 x 10^6 cells were lysed in 80% methanol. After centrifugation at 12000 r.p.m. for 10 minutes, the supernatants were collected and dried. Pellets were resuspended in water and analyzed using Acquity Ultra Performance Liquid Chromatography (UPLC, Waters) interfaced with a PDA photodiode array (Waters).

**Statistics**

Statistical analysis was performed using the unpaired, two-tailed Student’s t test or one-way ANOVA and Tukey’s test. The quantification for percentage of cells with cytoophidia was done with at least three repeat experiments, and more than 100 cells were counted for each quantification. All error bars showed in graphs represent standard error of mean.

**RESULTS AND DISCUSSION**

**CTPS and IMPDH form two types of cytoophidia**

CTPS and IMPDH are identified as major components of cytoophidia in mammalian cells (Carcamo et al., 2011; Chen et al., 2011). As shown in a recent study, CTPS and IMPDH can form two independent types of cytoophidium structure (Keppeke et al., 2015). To determine if CTPS and IMPDH cytoophidia are co-regulated or respond to
different stimuli, we firstly cultured human HEK 293T cells with medium containing a glutamine analog, DON, which blocks CTP and GTP biosynthesis, or an IMPDH specific inhibitor, MPA. Both DON and MPA can induce cytoophidium assembly (Carcamo et al., 2011; Chen et al., 2011; Ji et al., 2006; Keppeke et al., 2015). Under normal culture conditions, IMPDH cytoophidia were observed in about 30% of cells, whereas CTPS cytoophidia were hardly detectable (Fig. 1A, E, F). After treatment with DON for 1 day, the number of cells with CTPS and IMPDH cytoophidia significantly increased to over 80% and 90%, respectively (Fig. 1B, E, F). CTPS and IMPDH cytoophidia were observed as separate structures, but were sometimes fully or partially colocalized in the cytoplasm and the nucleus (Fig. 1B, D). When cells were treated with MPA, IMPDH cytoophidia formed in more than 90% of cells. Under the same conditions, more than 20% of cells also expressed CTPS cytoophidia (Fig. 1C, E, F). In our previous work, the CTPS1-GFP stably transfected HEK 293T cell line (CTPS1 OE) was established (Aughey et al., 2014). It has been shown that CTPS tends to aggregate in vitro when CTP and CTPS concentrations are high (Barry et al., 2014). Therefore, a 5-fold increase in CTPS1 protein level resulted in the induction of large CTPS cytoophidia in more than 80% of CTPS1 OE cells (Sup. Fig. 1B-F). We then perform immunostain on CTPS1 OE cells to check if regulation of IMPDH cytoophidia was affected by CTPS1 overexpression. Interestingly, a significant increase in cells with IMPDH cytoophidia was found for this cell line, up to more than 60%, with no changes in IMPDH expression level (Sup. Fig. 1A, C-F). Similar to DON-induced cytoophidia, some IMPDH cytoophidia partially colocalized with CTPS cytoophidia. This was also presented in cells transfected with a construct
encoding CTPS1 protein without GFP tag, excluding the possibility of the side effect resulted by chimeric recombinant protein (Sup. Fig. 1H-M). Furthermore, the nucleotide analysis shows that the GTP level was not changed in CTPS1 OE cells, indicating that these IMPDH cytoophidia were not induced by a GTP-deficient cell status (Sup. Fig. 1G), which was suggested as the condition that stimulates IMPDH cytoophidium formation (Calise et al., 2014). This result raised the possibility of that a 40% increase of CTP level in CTPS1 OE cells promotes IMPDH cytoophidium assembly. We then enlarged the intracellular CTP pool by supplementation of cytidine, which can be converted into CTP through a salvage pathway, and found that the percentage of cells with IMPDH cytoophidia slightly increased (Fig. 1G-K). As reported previously, the cytoophidium could generally be divided into “mature” and “immature” cytoophidium by their appearance (Calise et al., 2014). Several punctate and small spicule-shaped immature cytoophidia can form within a single cell, and they can undergo serial fusions to form larger ones (Gou et al., 2014; Thomas et al., 2012). On the other hand, only one or a few large linear or ring-shaped mature cytoophidia were presented by one cell in most cases (Fig. 1I). Under treatment with cytidine, more cells were found with mature IMPDH cytoophidia, and these were widely found when cells were treated with inhibitors blocking GTP synthesis (Fig. 1L).

**Inhibition of de novo CTP synthesis promotes IMPDH cytoophidium formation**

Purine and pyrimidine biosynthetic pathways are reciprocally regulated through sharing the pool of phosphoribosyl pyrophosphate (PRPP), which is the common
substrate of nucleotide synthetic pathways. CTP functions as a negative regulator of multiple steps in the de novo pyrimidine synthetic pathway (Lipscomb, 1994; Long and Pardee, 1967). We speculated that inhibition of pyrimidine biosynthesis by elevated CTP level, which activates de novo purine synthesis, promotes IMPDH cytoophidium assembly. Thus, we next treated HEK 293T cells with 3’-deazaauridine (DAU), an analog of uridine that can inhibit CTP biosynthesis by competitive inhibition of CTPS (Moriconi et al., 1986). During the first 1-hour period of DAU treatment, percentages of cells with IMPDH cytoophidia gradually increased, and more mature IMPDH cytoophidia were observed as well (Fig. 2A-C, F, G). However, numbers of cells with IMPDH cytoophidia declined with prolonged DAU treatment. At 4-hour of DAU treatment, only less than 20% of cells were found with immature IMPDH cytoophidia, and mature IMPDH cytoophidium was barely detectable (Fig2. D-G). Meanwhile, the number of CTPS cytoophidia gradually increased under the same condition (Fig. 2A-E). To check if IMPDH cytoophidium formation correlates with increased GTP synthesis, we traced nucleotide levels within the period of DAU treatment. The result shows that while the CTP level gradually decreased to less than half of the original level, GTP level doubled at the point of 2-hour DAU treatment (Fig. 2H). Additionally, the rate for GTP accumulation in the initial 1 hour was more than twice of the rate in later few hours of DAU treatment (Fig. 2I). It is known that guanine nucleotide inhibits multiple enzymes in de novo GTP synthetic pathway including PRPP synthetase, glutamine-PRPP amidotransferase and IMPDH (Lehninger et al., 2013). This provides the possible explanation for the decrease of IMPDH cytoophidia and gradually slowed accumulation of GTP within 1 to 4 hours.
of DAU treatment. We also treated HeLa cells with same conditions, and observed the similar phenomenon (Sup. Fig. 2). These results indicated that inhibition of CTP synthesis with DAU activates purine nucleotide synthesis in human cells and promotes IMPDH cytoophidia assembly.

Inhibition of cell growth triggers disassembly of IMPDH cytoophidia

In a screening of mammalian cell lines, we found that mature IMPDH cytoophidia were normally expressed in a mouse fetal liver cell line, BNL CL2. Under normal culture conditions, IMPDH cytoophidia spontaneously form in about 60% of BNL CL2 cells (Fig. 3A). Yet, only 22% (27/121) of cells undergoing mitosis contained detectable IMPDH cytoophidia. When cells launch division, they have to increase nucleotide production before or during S phase to meet the needs of protein expression and DNA replication. To test the correlation of IMPDH cytoophidia and cell growth, we cultured BNL CL2 cells in serum-free medium for 1 day. The percentage of cells with IMPDH cytoophidia dropped to only 20%, suggesting that active signaling pathways for cell growth are important for maintaining the IMPDH cytoophidium structure (Fig. 3B, E). Moreover, we also treated BNL CL2 cells with a PI3K inhibitor, LY294002, and rapamycin, a mTOR inhibitor, in order to restrict cell growth by blocking the PI3K/AKT/mTOR pathway. Inhibition of PI3K/AKT/mTOR pathway was confirmed by decreased level of phosphorylation at S473 of AKT, which indicates active PI3K signaling (Fig. 3F). The disturbance of cell cycle progression by inhibitors was also confirmed by flowcytometry (Sup. Fig. 3A). Consistently, IMPDH cytoophidia were barely observed upon inhibition of the pathway, while the IMPDH
protein expression level was not remarkably changed (Fig. 3C-F). Similar results were observed in HEK293T cells. When HEK 293T cells were cultured in medium containing LY294002 or rapamycin for 6 hours, the percentages of cells with IMPDH cytoophidia significantly dropped, and additional DAU in the last 1 hour of treatments failed to stimulate IMPDH cytoophidium assembly (Sup. Fig. 3B-F). These results again support our hypothesis that upregulation of purine nucleotide synthesis promotes IMPDH cytoophidium assembly (Fig. 3G).

**IMPDH forms cytoophidia in mouse pancreatic islet cells**

To determine if IMDPH forms cytoophidia *in vivo*, we used an antibody against IMPDH to stain sections of multiple mouse tissues, including intestine, stomach, skin, thymus, lymph node, brain, liver, testis and pancreas. We found that IMPDH cytoophidia were detectable in only a few cells in most tissues, except in pancreas, where we could detect very abundant IMPDH cytoophidia. When co-stained with the anti-insulin antibody, IMPDH cytoophidia were observed in many β cells (Fig. 4A-D). The secretion of insulin is tightly controlled by a combination of metabolic coupling factors; for example, insulin exocytosis associated GTPase enzymes utilize GTP to promote insulin secretion, and an increased GTP level was found at high glucose concentration (Robertson et al., 1991; Zou et al., 2014). Other studies have showed that treatment of MPA or ribavirin impeded the release of insulin in rat islets, and this inhibition could be eliminated by guanine supplementation, indicating that sufficient GTP is essential for insulin secretion (Kowluru et al., 1996; Metz et al., 1992). Accordingly, we propose that IMPDH filamentation in pancreatic β cells is correlated
with insulin secretion. To confirm this, we performed immunostain on tissues sections of pancreas collected from mice that were fasted overnight (18 hours) to keep its blood glucose concentration low. Consequently, IMPDH cytoophidia reduced in most islets of fasted mice (Fig. 4E-H). We also performed whole-mount stain on pancreatic islets freshly isolated from mice. Consistently, many IMPDH cytoophidia were observed in freshly isolated islets (Sup. Fig. 4A-C). To reduce glucose-stimulated insulin secretion, we further cultured isolated islets in HBSS as a nutrient deficient condition for 3 hours prior fixation. As expect, disassembly of IMPDH cytoophidia was shown in most islet cells (Sup. Fig. 4D-F). These results show that the regulation of IMPDH cytoophidium in pancreatic islet may respond to nutrient uptake of the cell.

In the past, most IMPDH cytoophidium studies were done with IMPDH inhibitors or glutamine analogs. Since such inhibitors directly disturb GTP biosynthesis, it is hard to determine the real regulation and function of the IMPDH cytoophidium under those conditions. Here, we show that assembly of the IMPDH cytoophidium is promoted by active regulation of purine synthesis in mammalian cell lines. One previous structural study has demonstrated that filamentation of human IMPDH1 protein occurs in the presence of its effector MgATP (Labesse et al., 2013). This is consistent with our findings. Although most of previous descriptions about IMPDH cytoophidium were focused on IMPDH2, the possibility of cross-reaction between two IMPDH isoforms was not excluded due to their high sequence similarity (Calise et al., 2014; Keppeke et al., 2015; Natsumeda et al., 1990). In this study, our immunostaining results were acquired with a mouse monoclonal antibody for human IMPDH1 (ab55297, abcam),
and two other anti-human IMPDH1 polyclonal antibodies (ab84957 and ab89048, abcam) were also tested. Because staining with three antibodies represented identical pattern, we could not rule out the possibility that both isoforms were under our observation.

It has been shown that CTPS cytoophidium acts as a negative regulator of CTP synthesis (Barry et al., 2014). Therefore, IMPDH and CTPS cytoophidia may play distinct roles in coordinating the balance of nucleotide pools. Further research is required to reveal how cytoophidium affects the enzymatic activity of IMPDH. Since IMPDH and CTPS have been considered as drug targets in the case of several types of disease, our results not only provide valuable information for understanding the putative roles of cytoophidia in cell metabolism, but also support future studies to determine the potential of the cytoophidium as a biomarker or target in medical applications.

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AUTHOR CONTRIBUTIONS

C.C.C., L.Y.S. and J.L.L. conceived this project; C.C.C. designed and performed the experiments; L.M.P and W.C.L helped with the nucleotide analysis; C.C.C., L.Y.S., J.L.L., H.S.L., S.T.D and S.C.W. analyzed the data; C.C.C. wrote the manuscript with input from L.Y.S. and J.L.L.

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Figure 1. CTPS and IMPDH can form independent cytoophidium structures.

Immunofluorescence of (A) untreated HEK 293T cells, and (B) HEK 293T treated with DON (10 μg/ml) or (C) treated with MPA (10 μM) for 1 day before fixation. Arrowheads indicate CTPS cytoophidia. Scale bar = 20 μm. (D) Magnified views of the boxed areas presented in (B) show colocalization of CTPS and IMPDH cytoophidia in the cytoplasm and the nucleus. Scale bar = 5 μm. (E) and (F) Quantification of percentages of cells with IMPDH and CTPS cytoophidia is shown (t test). (G-I) Immunofluorescence of HEK 293T cells cultured in medium with 100 μM cytidine for 1 hour before fixation. Mature and immature IMPDH cytoophidia are demonstrated in selected areas in (G). Percentages of cells with CTPS and IMPDH cytoophidia are shown in (J) and (K). There is no significant difference between the control and cytidine-treated group. (t test, p value = 0.16 and 0.19). (L) Ratios of mature and immature IMPDH cytoophidia observed in cells cultured under normal culture conditions (control), treated with 100 μM cytidine for 1 hour (+cytidine), and treated with 10 μM MPA for 1 day (+MPA).
Figure 2. Inhibition of de novo CTP synthesis promotes IMPDH cytoophidium assembly. (A-E) Immunofluorescence of HEK 293T cells treated with 100 μM 3’-deazauridine (DAU) for 0, 0.5, 1, 2 and 4 hours. Scale bar = 20 μm. (F) Percentages of cells with IMPDH cytoophidia (Tukey’s test). Ratios of mature and immature IMPDH cytoophidia observed in cells cultured in 100 μM DAU for different durations are shown in (G). (H) Nucleotide levels in cells treated with 100 μM DAU for different durations. Nucleotide concentration of each point was standardized by [ATP]. (I) The changes of GTP concentration with 1 hour shows that intracellular GTP accumulates faster in the first hour than later few hours of DAU treatment.
Figure 3. IMPDH forms cytoophidia in specific cell types *in vitro*. (A-D) Immunofluorescence of mouse BNL CL2 cells: untreated (control), treated, cultured with serum-free medium for 1 day, and treated with PI3K inhibitor, 50 μM LY294002, or mTOR inhibitor, 1μM rapamycin for 6 hours before fixation. Scale bar = 20 μm. (E) Percentages of cells with IMPDH cytoophidia after culture under various conditions (t test). (F) Immunoblotting of BNL CL2 cells cultured under various conditions. (G) A model for the regulation of IMPDH cytoophidium assembly.
Figure 4. IMPDH forms cytoophidia in mouse pancreatic islet cells.

(A-C) Immunofluorescence on a normal mouse pancreas section shows abundant immature IMPDH cytoophidia were expressed by islet cells. Scale bar = 20 μm. (D) Magnified view of boxed area present in (C). Scale bar = 5 μm. (E-G) Immunofluorescence on the section of pancreas from a fasted mouse shows the number of IMPDH cytoophidia in islet cells reduced. Scale bar = 20 μm. (H) Magnified view of boxed area present in (G). Scale bar = 5 μm.