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Assembly of IMPDH2-Based, CTPS-Based, and Mixed Rod/Ring Structures Is Dependent on Cell Type and Conditions of Induction

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ABSTRACT

Inhibition of guanosine triphosphate (GTP) and cytidine triphosphate (CTP) biosynthetic pathways induces cells to assemble rod/ring (RR) structures, also named cytoophidia, which consist of the enzymes cytidine triphosphate synthase (CTPS) and inosine-5′-monophosphate dehydrogenase 2 (IMPDH2). We aim to explore the interaction of CTPS and IMPDH2 in the generation of RR structures. HeLa and COS-7 cells were cultured in normal conditions or in the presence of 6-diazo-5-oxo-L-norleucine (DON), ribavirin, or mycophenolic acid (MPA). Over 90% of DON-treated cells presented RR structures. In HeLa cells, 35% of the RR structures were positive for IMPDH2 alone, 26% were CTPS alone, and 31% were IMPDH2/CTPS mixed, while in COS-7 cells, 42% of RR were IMPDH2 alone, 41% were CTPS alone, and 10% were IMPDH2/CTPS mixed. Ribavirin and MPA treatments induced only IMPDH2-based RR. Cells were also transfected with an N-terminal hemagglutinin (NHA)-tagged *CTPS1* construct. Over 95% of NHA-CTPS1 transfected cells with DON treatment presented IMPDH2-based RR and almost 100% presented CTPS1-based RR; when treated with ribavirin, over 94% of transfected cells presented IMPDH2-based RR and 37% presented CTPS1-based RR, whereas 2% of untreated transfected cells presented IMPDH2-based RR and 28% presented CTPS1-based RR. These results may help in understanding the relationship between CTP and GTP biosynthetic pathways, especially concerning the formation of filamentous RR structures.

KEYWORDS: Nucleotide synthesis; Enzyme aggregation; Enzyme inhibition; IMPDH2; CTPS

INTRODUCTION

The cytoplasm of eukaryotic cells has several organelles surrounded by membranes, such as the Golgi complex, mitochondria, and the endoplasmic reticulum. The segregation of cellular structures by membranes aims to generate and maintain controlled concentrations of different molecules at specific sites in the cell. Some cytoplasmic organelles are able to maintain themselves without the need for isolation membranes, such as ribosomes, proteasomes, cytoplasmic processing bodies (P bodies), uridine-rich small nuclear ribonucleoprotein bodies (U bodies), and purinosomes. Enzyme aggregation into non-membrane-bound bodies is a common feature in eukaryotic cells and numerous enzymes

Abbreviation: ANA-HEp-2, antinuclear antibody routine test using HEp-2 cells that originated from human laryngeal carcinoma; COS-7, African green monkey kidney fibroblast-like cells; CTPS, cytidine triphosphate synthase; DON, 6-diazo-5-oxo-L-norleucine; GFP tag, green fluorescent protein tag; GTP/CTP, nucleosides guanosine triphosphate/cytidine triphosphate; HeLa, human cervical cancer cells; HCV, hepatitis C virus; IF, immunofluorescence; IIF, indirect immunofluorescence; IMPDH2, inosine-5′-monophosphate dehydrogenase 2; MPA, mycophenolic acid; NHA tag, 22-amino-acid-long N peptide linked to the hemagglutinin tag.

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have been described to participate in this process (O'Connell et al., 2012).

In the last five years, many reports have described the ability of cytidine triphosphate synthase (CTPS) and inosine-5′-monophosphate dehydrogenase 2 (IMPDH2), that are critical in the cytidine and guanine nucleotide biosynthesis pathways, respectively, to form aggregates (Liu, 2010; Carcamo et al., 2011; Calise et al., 2014; Gou et al., 2014). Under special conditions, these enzymes aggregate into structures in the shape of 3–10 μm-long rods and 2–5 μm-diameter rings. These structures have received different designations, including Rods and Rings (or RR), cytoophidia (Greek for "cellular snakes"), and CTP synthase filaments (Carcamo et al., 2011, 2014; Liu, 2011). The rate-limiting step from both the *de novo* and the uridine salvage pathways in the synthesis of cytosine nucleotides is catalyzed by the key enzyme CTPS (Lieberman, 1956; van Kuilenburg et al., 2000). Interestingly, the catalysis of inosine-5′-monophosphate into xanthine-5′-monophosphate is done by IMPDH2, followed by a subsequent conversion into guanosine-5′-monophosphate. IMPDH2 is also involved in the control of the cellular pool of nucleotide guanine, necessary for DNA and RNA synthesis (Natsumeda et al., 1990; Bairagya et al., 2011). The formation of RR structures usually happens when the GTP/CTP pathways are inhibited, either by specific drug targeting of their enzymes or by lack of nutrients that would fuel these pathways. CTPS inhibitors, such as 6-diazo-5-oxo-L-norleucine (DON) and acivicin, and IMPDH2 inhibitors, such as ribavirin and mycophenolic acid (MPA), have been shown to induce RR structures (Carcamo et al., 2011; Chen et al., 2011). The culture of cells in medium without glutamine is also able to induce the cell to present RR structures, maybe because glutamine is an energy source for the GTP/CTP pathways (Calise et al., 2014; Gou et al., 2014).

The inhibition of GTP/CTP pathways is used as a therapeutic target in different clinical conditions. The anti-tumor efficacy of DON was confirmed in different animal models, but never approved as an anti-cancer drug (Yoshioka et al., 1992). MPA is used as an immunosuppressant for the prevention of organ transplant rejection and in the treatment of systemic lupus erythematosus (Moore and Derry, 2006; Knight et al., 2009). Ribavirin is used as an adjuvant to interferon-α therapy in chronic hepatitis C virus (HCV) infection. Curiously, it has been shown that 20%–40% of HCV patients treated with interferon-α plus ribavirin present autoantibodies against RR structures after six months of treatment, with titers increasing throughout treatment and eventually decreasing after interruption of treatment (Covini et al., 2012; Keppeke et al., 2012; Novembrino et al., 2014). These autoantibodies seem to be, in most cases, directed against IMPDH2, which is the very target of ribavirin used in the anti-HCV treatment (Carcamo et al., 2011, 2013; Seelig et al., 2011; Probst et al., 2013).

Most published studies on the induction of RR-like structures explored the inhibition of only a single enzyme, CTPS or IMPDH2. However, the study from Carcamo et al. (2011) showed both enzymes as components of RR structures. The present study aims to explore the interaction of CTPS and IMPDH2 enzymes in the generation of RR structures. To identify the contribution of each enzyme in individual RR structures, both enzymes were labeled with different fluorophores and evaluated in human and monkey cell lines under CTP/GTP pathway inhibition.

RESULTS

IMPDH2 and CTPS aggregation by treatment with GTP/CTP pathway inhibitors

The studies showed that IMPDH2 and CTPS enzymes can aggregate into RR structures under special circumstances, especially by interfering with GTP/CTP pathways (Ji et al., 2006; Gunter et al., 2008; Carcamo et al., 2011; Liu, 2011; Gou et al., 2014). In order to define the relative contributions of IMPDH2 and CTPS to RR structures, both enzymes were detected by immunostaining in HeLa and COS-7 cells treated with IMPDH2 (1 mmol/L ribavirin) or CTPS (1 mmol/L DON) inhibitors for 24 h. RR structures only recognized by anti-IMPDH2 antibodies were classified as IMPDH2-based RR, and those only recognized by anti-CTPS1 antibodies were classified as CTPS1-based RR. The majority of HeLa and COS-7 cells presented IMPDH2-based RR in cultures treated with either DON or ribavirin (over 92%, left column in Fig. 1). In contrast, CTPS1-based RR structures were observed in both cell lines but only in cells exposed to DON (over 94%, middle column in Fig. 1). Accordingly, the majority of DON-treated cells expressed both CTPS1-based and IMPDH2-based RR structures while ribavirin-treated cells only showed IMPDH2-based RR. As expected, only 4% of untreated HeLa cells showed RR (Fig. 1C and similar data not shown for COS-7).

As ribavirin is an irreversible inhibitor of IMPDH2, we also treated HeLa cells with the reversible IMPDH2-inhibitor mycophenolic acid (MPA) at 1 mmol/L for 24 h. Similar to ribavirin-treated, MPA-treated cells generated only IMPDH2-based RR structures (Fig. S1A). As previously reported (Gou et al., 2014), both CTPS1 and CTPS2 enzymes can form RR structures as demonstrated in HeLa cells with GFP fused to either human CTPS1 or CTPS2. In support of this observation, DON-treated HeLa and COS-7 cells were labeled with anti-IMPDH2 serum and rabbit anti-CTPS2 antibodies. Anti-CTPS2 antibodies also labeled the CTPS-based RR structures in DON-treated cells (Fig. S1B–D). However, the possibility of cross-reactivity of anti-CTPS2 antibodies with the CTPS1 enzyme cannot be excluded completely since there is a high degree of sequence similarity between the two isoforms.

DON treatment induced mixed RR structures with variable content of IMPDH2 and CTPS

DON treatment induced cells to produce both CTPS-based and IMPDH2-based RR structures (Fig. 1A and D). The in-depth Z-stack analysis with confocal IF microscopy revealed that DON-treated HeLa cells double-labeled with anti-IMPDH2