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Identification of the Polyhydroxybutyrate Granules in Mammalian Cultured Cells

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Abstract

Poly-3-hydroxybutyrate (PHB) is a biological polyester present in bacteria and eukaryotic cells. Long-chain (or storage) sPHB (up to 100,000 residues) is typically present in PHB-accumulating bacteria and localized in specialized granules known as carbonosomes. In these organisms, sPHB plays a major role as carbon and energy storage. On the other hand, short-chain (or complexed) cPHB (10–100 residues) is present in eukaryotic organisms, including mammals as well as in many bacteria. Previous studies indicated that cPHB is localized in various subcellular compartments of the eukaryotic organisms. Here, we used fluorescent microscopy to directly investigate the localization of PHB in mammalian cells. PHB was visualized in cultured U87 cells using fluorescent probe BODIPY 493/503. Specificity of PHB staining was confirmed by markedly decreased fluorescence of samples treated with PHB-specific depolymerase (PhaZ7). We found that PHB is associated with granules, and that these PHB-enriched granules do not co-localized with mitochondria, lysosomes, or endoplasmic reticulum. These results suggest that, in mammalian cells, PHB can accumulate in the cytoplasm in granules similar to ‘energy storage’ carbonosomes found in PHB-accumulating bacteria.

Introduction

Poly-3-hydroxybutyrate (PHB) is a biological polymer made of many 3-hydroxybutyrate (3-HB) residues that belongs to the polyhydroxyalkanoates class [1]. It has been detected in a large variety of organisms ranging from bacteria to mammals [2]. PHB is studied in great details in PHB-accumulating bacteria that use this polymer as a carbon and energy storage [3]. PHB accumulates in these organisms under conditions where there is oversupply of carbon sources and lack of other nutrients. PHB accumulation occurs in specialized subcellular organelles – carbonosomes [4]. In addition to PHB, these organelles contain a set of proteins directly linked to PHB metabolism. The size of PHB accumulated in these granules can reach up to 100,000 3-HB units per single polymer. The total amount of accumulated PHB can be as high as 20% of the total dry weight of the cell. The pool of PHB which is associated with energy-storage function in PHB-accumulating bacteria is generally referred to as ‘storage’ PHB or sPHB. While sPHB is assumed to be primarily present in bacteria, another class of PHB has been reported to be ubiquitously present in many types of living organisms [2][5][6]. This separate class of PHB consists of polymers that range from

10 to 100 3-HB residues. This shorter polymer is primarily found complexed to other molecules, and thus it is termed 'complexed' or cPHB. Unlike sPHB, which plays a major role as energy and carbon source, the role of cPHB is assumed to be involved in regulation of various cell functions through modification of target molecules. It was demonstrated that a complex of cPHB with Ca^{2+} and inorganic polyphosphate is involved in the formation of ion-conducting channels in bacterial [7][8] and mitochondrial membranes [9]. Furthermore, cPHB can interact with membrane proteins through hydrophobic and perhaps covalent interactions [10][11]. It has been suggested that, in the case of protein channels, these interactions might play an important role in regulation of channel function and selectivity [12]. Previous studies indicate that cPHB can be found in various subcellular compartments of the eukaryotic organisms [6] as well as associated with specific proteins [2][13]. The aim of the present study was to directly investigate localization of PHB in cultured mammalian cells. Our results suggest that a significant amount of PHB is localized in granules. We hypothesize that these granules might be similar to carbonosomes found in PHB-accumulating organisms.

Results and Discussion

PHB Localization in Cultured Mammalian Cells

Previous reports of studies of PHB in bacteria indicate that in these organisms PHB can be visualized using fluorescent probe BODIPY 493/503 [14]. BODIPY 493/503 is a fluorescent probe which tends to localize in strongly hydrophobic environments. Due to the presence of a high number of hydrophobic groups and high flexibility of the PHB polymer, it can form highly hydrophobic aggregates, which provide a favorable environment for BODIPY 493/503. In previous studies, fluorescent staining of bacteria with probes similar to BODIPY 493/503 were used for detection of intracellular PHB-rich organelles [15]. In addition to PHB, these organelles, named carbonosomes, contain a number of proteins involved in PHB metabolism [4]. Here, we used BODIPY 493/503 to investigate the distribution and localization of PHB in mammalian cells. In our experiments, we used U87 human astrocytoma cell line. Fixed U87 cells incubated with BODIPY 493/503 showed intense green granules of variable sizes in the cytoplasm of both live (Fig. 1, *a–c*) and fixed cells (Fig. 1, *d–f*). Although, these data support the idea of the presence of PHB vesicles, it is known that BODIPY 493/503 can stain also other lipophilic granules. Notably, it is widely used to visualize lipid droplets enriched in triglycerides and cholesteryl ester found in many cell types [16]. Thus, to confirm that BODIPY 493/503 staining indeed reflects the presence of PHB, fixed U87 were incubated with a PHB depolymerase, PhaZ7. As a control, we used a point mutant form of PhaZ7 (PhaZ7 S136A) which lacks PHB-depolymerizing activity [17]. Enzyme treatment was followed by staining with BODIPY 493/503. When the cells were treated with PhaZ7, the staining of these granules decreased (Fig. 2, *b* and *e*) compared to the fixed cells treated with the mutant PhaZ7 S136A (Fig. 2, *c* and *f*), and to non-treated controls (Fig. 2, *a* and *d*). Since PhaZ7 depolymerase is specific for the native form of the polymer, these results support the idea that the vesicles contain PHB [17][18].

Investigation of the Association of PHB Vesicles with Other Cellular Organelles

To analyze if PHB-enriched granules were co-localized with different organelles of the cell, we used BODIPY 493/503 staining in combination with fluorescent probes—markers of the various cellular organelles. We used TMRM to visualize mitochondria of live cells, immunofluorescence approach to localize endoplasmic reticulum (ER) utilizing an antibody against vesicle-associated membrane protein-associated protein-A (VAP) and lysotracker to identify lysosomes. Fig. 3 shows co-staining of live U87 cells with BODIPY 493/503 and TMRM probe. TMRM is a charged red fluorescent probe which in live cells localizes primarily in the mitochondria. As can be seen from the overlay image as well as from

overlay of fluorescence intensity profiles, PHB vesicles are not localized in the mitochondria. Furthermore, the endoplasmic reticulum (Fig. 4), visualized using ER-specific antibodies, also showed no co-localization with the PHB granules. The localization relative to lysosomes of these PHB-enriched vesicles was studied in live cells with markers for lysosome (lysotracker; Fig. 5). As can be seen from this figure, PHB granules do not co-localize with lysosomes.

Our study identifies the presence of the PHB-rich granules in mammalian cells. At present, it is not clear whether these granules represent unique organelles or localized in some previously described subcellular structures. It should be noted that other subcellular vesicle – lipid droplets – can be stained with BODIPY 493/503. However, lipid droplets are composed primarily of triacylglycerols and sterol esters, and it is unlikely that their morphology will be affected by PhaZ7 treatment. This suggests that PHB granules seen in our experiments are structures which are different from lipid droplets.

While physiological role of PHB granules remains to be established, it is tantalizing to propose that, similar to bacteria, mammalian cells might contain PHB-rich granules which serve as energy reserves. PHB is composed of HB residues which are also known as ketone bodies. It is possible that the amount, size, and distribution of PHB granules are directly linked to the levels of glucose, fatty acids, and ketone bodies as an energy substrates of the cell. According to this scenario, depending on the specific conditions, cells might be able to accumulate or consume PHB associated with these vesicles.

Experimental Part

Cell Line

U87 Cells were maintained in high glucose *Dulbecco's* modified *Eagle's* medium (DMEM; *Gibco*, Cat # 11995) supplemented with 10% FBS (*Invitrogen*, Cat # 12483) and 1% penicillin/streptomycin (*Invitrogen*, Cat #). Cells were cultured at 37° in a humidified tissue culture incubator containing 5% CO₂ in air.

Reagents

4,4-Difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-*s*-indacene (*BODIPY*[®] 493/503 Cat # D3922) and *LysoTracker*[®] (Cat # L7528) were purchased from *Molecular Probes* (*Invitrogen*, Oregon, USA). Anti-VAP antibody, a marker for endoplasmic reticulum, was a gift from Dr. *Ridgway* (Wyles JP 2002). *Dylight 549* donkey anti rabbit (Cat #711-505-152), *Dylight 659* anti-mouse (Cat # 115-495-003), and *Dylight 549* anti-mouse (Cat#715–505–150) conjugated secondary antibodies were purchased from *Jackson ImmunoResearch Laboratories* (West Grove, PA, USA). Native polyhydroxybutyrate depolymerase 7, PhaZ7 WT, and a point mutant form of the depolymerase, PhaZ7 S136A, with no enzymatic activity were expressed in *E. coli* and were purified from the supernatants with ion-affinity chromatography as described in [19] (clones were kindly provided by Dr. *D. Jendrossek*).

Immunocytochemistry

U87 Cells were fixed in 4% paraformaldehyde (PFA) for 30 min, washed in PBS and treated with 100 mM glycine for 30 min. This was followed by three washings with PBS and a permeabilization step with MeOH at –20° for 5 min. The fixed cells were blocked with 1.5% gelatin in PBS for 1 h at r.t. and incubated with anti-VAP for 1 h. Following 3 washings with PBS, the secondary conjugate antibody was incubated for 1 h and washed with PBS.

BODIPY Staining

Fixed cells were incubated with BODIPY 493/503 (working concentration, 0.1 $\mu\text{g/ml}$) for 2 min and washed with HBSS-buffered salt soln. (5.33 mM KCl, 0.44 mM KH_2PO_4 , 4.17 mM NaHCO_3 , 137.93 mM NaCl, 0.33 mM Na_2HPO_4 , 5.56 mM glucose, 1 mM MgCl_2 , 1 mM CaCl_2 , pH 7.44). When necessary, cells were incubated with 25 nM TMRM for at least 30 min before the experiment.

PHB Depolymerase Treatment

Fixed U87 were treated with PhaZ 7 WT, PhaZ 7 S136A, or *Tris* buffer (100 mM *Tris*, 1 mM MgCl_2 , 1 mM CaCl_2 , pH 9) as a non-treatment control for 1 h at 37°. After washings, cells were loaded with BODIPY (0.1 $\mu\text{g/ml}$) for 2 min, washed, and images were obtained using an *Axiovert 200M* inverted fluorescent microscope attached to a *Hamamatsu Orca R2 Camera* (Zeiss, Thornwood, NY).

Confocal Microscopy

Images were obtained using a *510 CLSM* (Zeiss, Thornwood, NY) equipped with a META detection system and a *X40* oil-immersion objective. The 488-nm Ar laser line was used to excite BODIPY 493/503 which was measured using a bandpass filter from 505–550 nm. Illumination intensity was kept to a minimum (at 0.1–0.2% of laser output) to avoid phototoxicity, and the pinhole was set to give an optical slice of *ca.* 1 μm . TMRM, *Dylight 549*, and lysotracker were excited with the 543-nm laser line, and fluorescence was measured using a 560-nm longpass filter. When *Dylight 649* was used, the filters were set to a bandpass of 560–615 nm for red and longpass 650 nm for far-red.

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Fig. 1. BODIPY Staining in fixed and live U87 cells

Cells were either incubated 2 min with 0.1 $\mu\text{g/ml}$ BODIPY 493/503 in HBSS, pH 7.4, washed, and imaged (*a-c*), or fixed with 4% PFA, washed, permeabilized with cold MeOH, and stained with 0.1 $\mu\text{g/ml}$ BODIPY 493/503 (*d-f*). Scale bars, 10 μm .

Fig. 2. PhaZ7 WT Hydrolase PHB granules stained with BODIPY

Fixed U87 were treated with buffer alone (*a* and *d*), PHAZ7 WT (*b* and *e*), or PhaZ7 S136A (*c* and *f*) in 100 mM *Tris*, 1 mM MgCl_2 , 1 mM CaCl_2 , pH 9, for 1 h, washed in HBSS with 1 mM CaCl_2 ; 1 mM MgCl_2 , and stained with BODIPY 493/503 (0.1 $\mu\text{g/ml}$) for 5 min, washed and imaged with an *Axiovert Zeiss* inverted fluorescence microscope. Scale bars, 20 μm .

Fig. 3. Mitochondria and lipid droplets

U87 Cells in HBSS media were loaded with 0.1 $\mu\text{g/ml}$ BODIPY (a) and 25 nM TMRM (b). Images were merged with *Zeiss LSM 510* software, and intensity profiles were collected along the white axis shown in c. Scale bars, 10 μm .

Fig. 4. Endoplasmic reticulum and lipid droplets

Fixed U87 cells stained with anti-VAP (*b*) were loaded with 0.1 μg/ml of BODIPY in HBSS (*a*). Images were merged with *Zeiss LSM 510* software, and intensity profiles were collected along the white axis shown in *c*. Scale bars, 10 μm.

Fig. 5. Lysosomes and lipid droplets

U87 Cells in HBSS were loaded with 0.1 $\mu\text{g/ml}$ BODIPY (a) and 100 nM lysotracker (b). Images were merged with *Zeiss LSM 510* software, and intensity profiles were collected along the white axis shown in c. Scale bars, 10 μm .