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Analysis of Phosphoinositide Dynamics During Phagocytosis Using Genetically Encoded Fluorescent Biosensors

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Summary

Phosphoinositide signaling is essential for successful phagocytosis. Phosphoinositides regulate processes such as actin assembly and the recruitment of molecular motors required for ingestion, as well as fusion events required for the maturation of the phagosome. Phosphoinositides not only serve as substrates for the generation of second messengers, but also function to anchor to the membrane cytosolic proteins that contain phosphoinositide-binding motifs. Conventional methods for the detection of phosphoinositides involve their extraction from the cells and separation by chromatographic procedures. These approaches are laborious and expensive and fail to provide spatio-temporal information, which is critical when analyzing localized and transient phenomena like phagocytosis. In this chapter we describe a method to monitor phosphoinositides dynamically by transfection of fluorescently tagged probes (biosensors) into cultured macrophages. These biosensors are based on the fusion of phosphoinositide-binding protein domains with fluorescent proteins. Some specifications for live cell imaging of such phosphoinositide-specific probes are also provided.

Key Words: Phosphoinositide; phagocytosis; phagosome; macrophage; PH domain; confocal microscopy.

1. Introduction

Phagocytosis is defined as the engulfment of large particles, greater than 0.5 μm in diameter, by specialized cells such as neutrophils and macrophages. Phagocytosis is essential for the destruction of pathogens, the clearance of apoptotic cells, and tissue remodeling ***(1)***. The phagocytic process comprises

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a variety of events that are initiated by the engagement of surface receptors and involve multiple signaling pathways. The number and nature of the signals elicited vary depending on the type of receptor engaged. A wide variety of phagocytic receptors have been described, among which the FcR and the CR3 receptors are the most thoroughly characterized. Receptor engagement and the ensuing signals ultimately lead to internalization of the particle into a membrane-bound vacuole or phagosome. The signaling cascade triggered by the activated receptors usually involves activation of protein kinases, alterations in phospholipid metabolism, remodeling of the actin cytoskeleton, and localized acceleration of membrane traffic ***(2)***.

Upon phagocytosis, the invading organism or alien particle is confined in the phagosome, which then undergoes a process of maturation. During the course of maturation the phagosome interacts with components of the endocytic pathway by a series of fusion and fission events that ultimately yield a hybrid organelle, the phagolysosome ***(3)***. Throughout this remodeling process the phagosome acquires degradative properties such as an acidic luminal pH, the delivery and activation of hydrolytic enzymes, and the production of reactive oxygen species that are essential to its microbicidal function ***(3)***.

Recent studies indicate that phosphoinositides play various, important roles in phagosome formation and maturation. Phosphoinositides are derivatives of the lipid phosphatidylinositol and are thought to be found in virtually all cellular membranes ***(4)***. Polyphosphoinositides can be reversibly phosphorylated on positions 3, 4, and/or 5 of the inositol head group, resulting in the generation of seven different species ***(5–8)***. In addition to their role as substrates for the phospholipases that generate second messengers, phosphoinositides also act themselves as membrane anchors for cytosolic proteins that possess a cognate phosphoinositide-binding domain ***(9–11)***. As a result, changes in phosphoinositide composition provide signals for the selective recruitment and activation of signaling proteins to specific membranes in the cell ***(4)***.

Phosphoinositides have been shown to be involved in signaling the early stages of phagocytosis, leading to actin assembly and the recruitment of molecular motors to the site of ingestion ***(1)***. They also regulate multiple budding, fission and fusion events required for maturation ***(1)***. Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), which is constitutively present in the plasma membrane, becomes transiently and modestly accumulated in pseudopods of forming phagosomes, but disappears rapidly thereafter ***(1)***. At least two reactions contribute to the disappearance of $PI(4,5)P_2$: its degradation by PLCγ, with the concomitant production of diacylglycerol (DAG) and inositol trisphosphate (IP3) ***(11)***, and its conversion to phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) by class I phosphatidylinositol 3-kinase (PI3K) ***(2)***. $PI(4,5)P_2$ itself can promote actin polymerization, and its disappearance is

therefore important for the termination of actin assembly ***(12)***. On the other hand, PI(3,4,5)P_3 undergoes a significant accumulation at sites of phagosome formation and remains briefly associated with nascent phagosomes ***(2,13)***. Newly formed PI(3,4,5)P_3 in turn stimulates the hydrolysis of PI(4,5)P_2 by promoting the recruitment and activation of PLCγ ***(14)***. PI(3,4,5)P_3 also contributes directly to the mechanical events that lead to particle intake by recruiting WAVE and myosin X to the phagosome ***(15)***. In addition, 3-phosphoinositides seem to be involved in the fusion of endomembranes at sites of phagocytosis, which promotes the conservation of the cell surface area ***(16)***. 3-Phosphoinositides, particularly phosphatidylinositol 3-phosphate (PI(3)P), appear to be crucial for phagosome maturation. PI(3)P accumulates on the phagosomal membrane within 1–2 min of sealing and persists for about 10 min ***(17)***. PI(3)P seems to be important for the recruitment of ligands such as EEA1 and Hrs, which are important for the fusion on membranes that bear Rab5 and Rab4 and for the inward membrane budding that generates multivesicular bodies ***(18)***.

To date, the majority of approaches to detect and measure the cellular content of phosphoinositides have relied on the use of radioisotope labeling, in which cellular lipids are metabolically tagged, followed by separation using laborious chromatographic procedures such as high-performance liquid chromatography (HPLC) and/or thin-layer chromatography (TLC) ***(19,20)***. Recently, mass spectrometry has also been utilized to measure phosphoinositides, but while this approach bypasses the need for isotopic labeling, its sensitivity is insufficient to detect several minor, yet physiologically very important inositide species ***(21)***. These methods are all extremely time-consuming and require the use of large numbers of cells and expensive reagents. Most importantly, because they are all end-point determinations, these approaches fail to reveal the spatio-temporal dynamics of phosphoinositides in the cell. These limitations were recently overcome by the introduction of fluorescently tagged phosphoinositide-specific probes that can be monitored continuously by noninvasive means. In essence, these novel probes consist of selected protein domains (modules) with well-defined phosphoinositide-binding properties, which are tagged with a fluorescent moiety. For convenience, fluorescent properties are conferred to these domains by fusion with a humanized version of the jellyfish green fluorescent protein (GFP), or any of its multiple variants (e.g., CFP, YFP), or with one of the red fluorescent proteins from other invertebrate species **(Fig. 1)**. In this manner, the distribution of the fluorescently labeled phosphoinositide-binding domains can be monitored by light microscopy in living cells. One of the major advantages of this approach is that the probe is genetically encoded and can be delivered into the cells by transfection, viral infection, or microinjection of the corresponding cDNA.

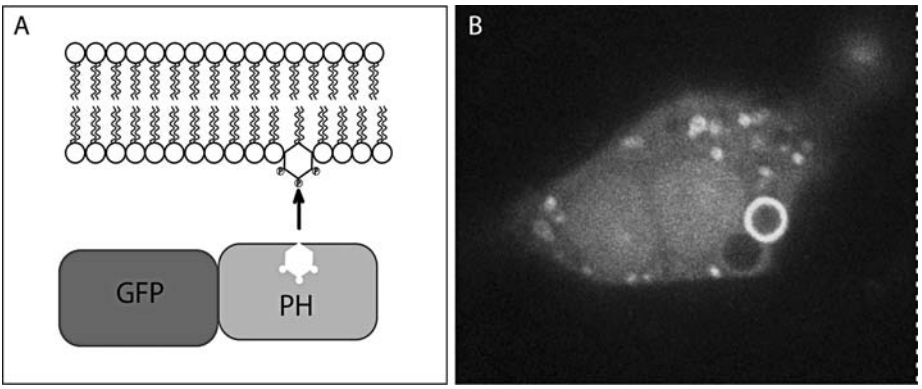

Fig. 1. Biosensor strategy used to detect phosphoinositides. **(A)** A chimeric construct is generated by fusing a well-defined phosphoinositide-binding motif, such as the PH domain illustrated here, and a fluorescent protein like GFP. Expression of the cDNA encoding for the chimera will generate a specific fluorescent probe detectable in live cells by noninvasive means. **(B)** Distribution of PI(3)P accumulation during phagosome maturation. PI(3)P was monitored by using the PX domain from p40phox ***(35)*** coupled to GFP. The present picture was taken 2 min after phagosome sealing. Notice that endosomes are also labeled by this chimeric construct due to their PI(3)P content.

The vast repertoire of known phosphoinositide-interacting proteins has led to the identification and characterization of a variety of phosphoinositide-binding modules with distinct specificities and affinities **(Table 1)**. The best characterized module is the PH (pleckstrin-homology) domain ***(22)***, which is present in proteins such as Akt/PKB, PDK1 and GRP1. More recently, a variety of other phosphoinositide-binding domains have been identified and characterized, including PX (Phox) ***(23)***, ENTH (epsin N-terminal homology) ***(24)***, FYVE (Fab1, YOTB, Vac1p, and EEA1) ***(25)***, FERM (Ezrin, Radixin, and Moesin) ***(26)***, and GRAM (glucosyltransferases, Rab-like GTPase activators, and myotubularins) ***(27)*** domains that are present in a variety of proteins.

The suitability of phosphoinositide-binding probes for live cell imaging has been the subject of recent debate. It is important to consider that, even though in vitro binding assays may indicate that a particular lipid-binding domain binds preferentially to one of several purified lipids, the probe may not show the same selectivity inside cells. Often cellular domains are coincidence detectors that sense two or more binding determinants simultaneously. While only lipids are present in the in vitro assays, other factors, such as proteins binding to other moieties of the probe are likely to exist in vivo, contributing to their recruitment ***(52)***. In this regard, it is desirable to validate the observations made with any one probe by other techniques or utilizing different lipid-binding

Table 1
Phosphoinositide-Binding Domains

Phosphoinositide	Protein domain	Ref.
PtdIns4P	FAPP1-PH	***(28–30)***
	OSH2-PH	***(31,32)***
	OSBP-PH	***(28,29,33)***
PtdIns3P	EEA1-FYVE, Hrs-FYVE	***(34)***
	p40phox-PX	***(35)***
PtdIns(4,5)P_2	PLCδ1-PH	***(36,37)***
	Tubby	***(38)***
PtdIns(3,4)P_2	TAPP1-PH	***(39)***
PtdIns(3,5)P_2	Svp1p	***(40)***
	Centaurinβ2-PH	***(41)***
PtdIns(3,4)P_2/	AKT-PH	***(42,43)***
PtdIns(3,4,5)P_3	PDK1-PH	***(44)***
	CRAC-PH	***(45)***
PtdIns(3,4,5)P_3	GRP1-PH	***(46,47)***
	ARNO-PH	***(48)***
	Btk-PH	***(49)***
	Cytohesin-1-PH	***(50,51)***

Source: Adapted from **ref.** ***(53)***.

domains that may have distinct binding determinants yet similar lipid selectivity in vitro. Furthermore, it is important to exercise care in the interpretation of results when using probes that can bind to more than one phosphoinositide species. In this case, as before, it is particularly helpful to confirm the findings using alternate probes. Finally, the level of expression of a given probe can be critical. Excessive expression of certain probes may result in interference with the cellular process they are supposed to monitor. This complication can be minimized by analyzing exclusively those cells with the lowest expression level compatible with adequate imaging and by comparing the phenotype of cells with varying levels of expression.

Introduction of the appropriate cDNA into phagocytes is essential for the expression of the biosensors and the subsequent imaging of phosphoinositide dynamics during phagosome formation and maturation. Primary professional phagocytes like macrophages and neutrophils are notoriously refractory to transfection or viral infection, so that heroic efforts are often required to introduce the plasmids. On the other hand, some cultured phagocytic cells, such as the mouse monocytic RAW 264.7 cell line, are transfectable at reasonable rates by either lipofection or electroporation. The use of RAW 264.7 cells for the dynamic study of phosphoinositide metabolism during phagocytosis is described below.

2. Materials

2.1. Cell Culture

1. Dulbecco's modified Eagle's medium 1X (DMEM) (cat. no. 319-005-CL. WISENT, Inc., Mississauga, ON) supplemented with 5% fetal bovine serum (FBS PREMIUM, cat. no. 080450 WISENT, Inc.).
2. Hydroxyethyl piperazine ethane sulfonate (HEPES)-buffered solution RPMI 1640 1X (HPMI) (Cat. No. 350-025-CL WISENT, Inc.).
3. Phosphate-buffered saline (PBS) (cat. no. 311-010-CL WISENT, Inc.).
4. Solution of Trypsin (0.05%) and ethylenediamine tetraacetic acid (EDTA) (0.53 m*M*) (cat. no. 325-042-EL WISENT, Inc.).
5. Round glass cover slips (25 mm diameter) (Fisher, Pittsburgh, PA).

2.2. Transfection Reagents

1. Cell line Nucleofector Kit V (cat. no. VCA-1003. Amaxa BioSystems. Cologne, Germany).

2.3. cDNA Preparation

1. HiSpeed Plasmid Maxi Kit (cat. no. 12663 QIAGEN Inc. Mississauga, ON).

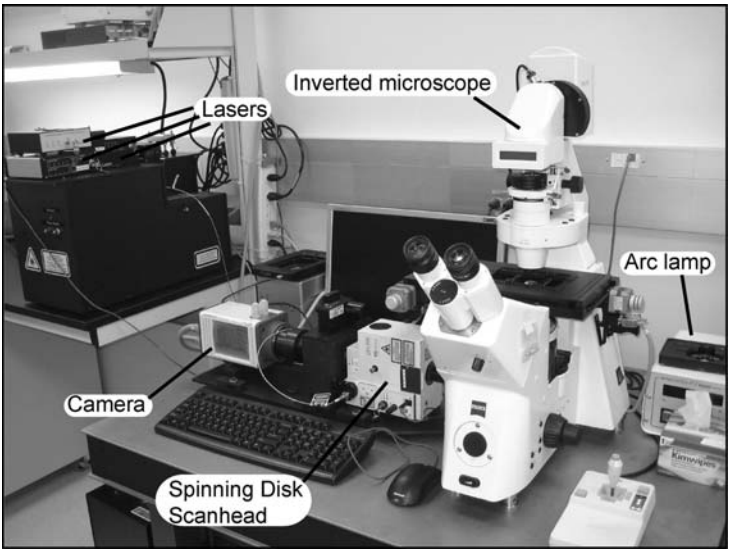

Fig. 2. Spinning disk confocal microscope. The system, such as the one illustrated, consists of a microscope with arc lamp, illumination lasers, spinning disk assembly, and detector cooled CCD camera, all driven by suitable software (e.g., Volocity) from a desktop computer. A system assembled and marketed by Quorum is shown here.

2.4. Particle Opsonization

1. Uniform latex microspheres (3.87 μm) (cat. no. PS05N, Bangs Laboratories, Inc., Fishers, IN).
2. Human IgG (Sigma-Aldrich, St. Louis, MO).

2.5. Confocal Microscopy

1. Quorum Spinning Disk Confocal Microscopy System (Quorum, ON, CA) (**Fig. 2**). Consists of a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu back-thinned EM-CCD camera and spinning disk confocal scan head. The unit is equipped with four separate diode-pumped solid-state laser lines (Spectral Applied Research, Richmond Hill, Ontario, Canada: 405, 491, 561, 652 nm), an ASI motorized XY stage, an Improvision Piezo Focus Drive and a 1.5X magnification lens (Spectral Applied Research). The equipment is driven by Volocity acquisition software and powered by an Apple Power Mac G5 computer.
2. Attofluor chamber (cat. no. A7816. Molecular Probes, Eugene, Oregon, Invitrogen).
3. Digital temperature regulator with a P-insert (PECON, Germany).

3. Methods

3.1. Cell Culture

RAW 264.7 macrophages from the American Tissue Culture Collection (ATCC) grown in DMEM plus 5% FBS are split at 70–80% confluency, diluted approximately 1:2 to 1:8 and seeded at a density of 2–4 × 10^4 viable cells/cm^2 in the same medium (*see* **Note 1**). Cells are removed by addition of trypsin and incubation at 37°C for 5–10 min.

3.2. Transfection

RAW 264.7 macrophages are transfected by electroporation using the Amaxa system (Cologne, Germany) (*see* **Note 2**) by following the manufacturer's instructions with some modifications:

3.2.1. Day 1

1. Cells are subcultured the day before transfection so that they are in a growth phase by the next day (approximately 70% confluence).

3.2.2. Day 2

1. Prewarm the Nucleofector solution (previously prepared by the addition of the "supplement solution") to room temperature. Prewarm an aliquot of culture medium (DMEM/5% FBS) to 37ºC.

2. Prepare a 6-well plate (Becton, Dickinson, Falcon cat. no. 353046) by placing one 25-mm sterile coverslip per well and adding 2 mL of DMEM/5% FBS. Place the plate in the tissue culture incubator.
3. Retrieve cells cultured from Day 1, rinse them once with PBS, and add 2 mL of the trypsin solution. Incubate cells with trypsin for about 5–10 min at 37°C (*see* **Note 3**). Stop trypsinization by adding 5 mL of DMEM/5% FBS. Take an aliquot of the cells and determine the concentration of viable cells by adding Trypan blue and counting the cells on a hemocytometer.
4. Centrifuge the required number of cells (2×10^6 cells per sample) at 100*g* for 10 min. Discard the supernatant.
5. Resuspend the pellet in Nucleofector solution pre-equilibrated to room temperature to a final concentration of 2×10^6 cells/100 µL. Add 2 µg of cDNA (final concentration 0.5–1 µg/µL), mix by pipetting and transfer the suspension to the electroporation cuvette. It is important to avoid maintaining the cell suspension longer than 15–20 min, since prolonged exposure to the Nucleofector solution reduces cell viability and gene transfer efficiency.
6. Insert the cuvette into the electroporator and apply program D-32 (*see* **Note 4**).
7. Remove the cuvette from the electroporator, add 500 µL of warm culture medium (DMEM/5% FBS) as soon as possible, remove the cells with a transfer pipet provided by the supplier, and seed them onto the prepared 25-mm coverslip, previously placed on the 6-well plate.
8. Place the plate in the tissue culture incubator. Depending on the construct utilized, expression is detectable after 6–24 h.

Generally, 40% of the surviving cells are transfected, although these results may vary with the particular gene expressed.

3.3. Opsonization of Latex Beads

1. Aliquot 200 µL of latex beads directly from the stock suspension (10% solids). Spin the beads in a microcentrifuge at maximum speed for 1 min, remove the supernatant, and wash the beads three times by resuspending them in 1 mL of PBS.
2. After the last wash, resuspend the beads in 300 µL of PBS and add 10 µL of human IgG (stock 2 mg/mL). Incubate for 1 h at room temperature with continuous rotation.
3. Wash 3 times in 1 mL of PBS. Resuspend beads in 1 mL of PBS after the last wash.

3.4. Phagocytosis Assay

1. Some time after transfection (6–24 h), the coverslip with adherent cells is removed from the culture plate and transferred to a clean "Attofluor" chamber and 1–1.5 mL of cold solution HPMI is added (*see* **Note 5**).
2. Add 50 µL of the opsonized latex bead suspension.

3. Place the chamber on ice and bring it to the spinning disk confocal microscope (*see* **Note 6**).
4. Place the chamber on the temperature regulator.
5. Locate the cells and identify the focal plane under bright field illumination by using 63X or 100X oil immersion objectives. Detect the transfected cells by epifluorescence using the binoculars and the appropriate fluorescence cube and switch immediately to confocal mode using the appropriate laser line and filter set of the spinning disk. Adjust the conditions for image acquisition, particularly the detector gain and amplifier offset, as well as the frequency and total time of acquisition (*see* **Note 7**).
6. Replace the medium for prewarmed serum-free HPMI and set the temperature regulator at 37°C. Initiate recording of fluorescence during the course of particle ingestion and/or maturation.

3.5. Fluorescence Measurements

To quantify the recruitment of the probes to a specific cell location, fluorescence intensity can be measured precisely by acquiring digital images, which are then analyzed with suitable software (*see* **Note 8**). Quantitation is performed by selecting the region of interest within the cell and comparing its intensity to the cytosolic fluorescence. To allow comparison among cells and between experiments, the background-corrected value is normalized for expression level by dividing it by the cytosolic fluorescence of the same cell.

4. Notes

1. RAW 264.7 cells should not be allowed to reach confluence, as this can lead to cell rounding and overcrowding, which reduces the phagocytic index and complicates the analysis of individual cells. Each batch of cells should be passaged a maximum of 20 times to avoid senescence.
2. In our hands, electroporation of RAW 264.7 macrophages using the Amaxa system is the method that yields the highest transfection efficiency. However, only a fraction of the cells subjected to electroporation survive the procedure, so a comparatively large number of cells need to be transfected in order to obtain a reasonable number of transfectants. Another complication is that the levels of expression of the transfected construct can be very high, potentially interfering or causing loss of cell viability. Other methods for delivery of cDNA that may be considered include the infection of cells with retroviral or lentiviral vectors. This approach is often efficient and yields high cell survival. The level of expression is in general more moderate, less likely to interfere with cell function. On the other hand, construction and packaging of viral vectors can be very laborious and expensive.
3. If the cells haven't detached completely from the plate after 10 min of incubation with trypsin, scrape them very gently after neutralizing the protease.

4. We have noticed that the application of program U-14 yields higher transfection efficiency than program D-32. However, the survival of the cells is reduced and 3 million cells should be used per transfection, instead of 2 million.
5. The incubation in cold medium slows down phagocytosis, which facilitates synchronization of the process.
6. The most common type of confocal microscope, the laser scanning microscope, uses a single focused laser beam that progressively rasters an entire region of interest ***(54)***. In contrast to the scanning microscope, the spinning disk confocal microscope uses a parallelized approach of multibeam scanning ***(54)*** by means of a rapidly spinning Nipkow disk with multiple pinholes in the light path, which results in the near-simultaneous excitation of the entire field of view. This enables the spinning disk system to acquire entire images at a very high speed, unlike the scanning laser microscope. In addition, the quantum efficiency of the spinning disk confocal detector (a CCD, or charge coupled device) is significantly higher than that of the photomultiplier tube used by the scanning microscope. Furthermore, since in the spinning disk confocal the excitation light is split into many mini-beams of correspondingly lower intensity, the overall photobleaching and phototoxicity is minimized, making it ideal for live cell, time-lapse imaging. However, it is important to mention that spinning disk microscopes are not equipped for regional bleaching that is required for photobleaching and photoactivation measurements.
7. For phagocytosis assays we usually acquire images every 5–10 s over a 5- to 7-min period.
8. Programs that can be used for this purpose include Image J, Inc. (freely accessible to the general public at http://rsb.info.nih.gov/ij/) or MetaMorph (Universal Imaging Corp.).

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