

Comparative characterization of phosphatidic acid sensors and their localization during frustrated phagocytosis

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ABSTRACT

Phosphatidic acid (PA) is the simplest phospholipid naturally existing in living organisms, but constitutes only a minor fraction of total cell lipids. PA has attracted considerable attention because it is a phospholipid precursor, a lipid second messenger and a modulator of membrane shape; and has thus, been proposed to play key cellular functions. The dynamics of PA in cells and in subcellular compartments, however, remains an open question. The recent generation of fluorescent probes for PA, by fusing GFP to PA-binding domains, has provided direct evidence for PA dynamics in different intracellular compartments. Here, three PA sensors were characterized *in vitro* and their preferences for different PA species in particular lipidic environments compared. In addition, the localization of PA in macrophages during frustrated phagocytosis was examined using these PA sensors and combined with a lipidomic analysis of PA in intracellular compartments. The results indicate that the PA sensors display some preferences for specific PA species, depending on the lipid environment, and the localization study in macrophages revealed the

complexity of intracellular PA dynamics.

INTRODUCTION

Phosphatidic acid (PA) has emerged as a new class of lipid mediators involved in diverse cellular functions through either structural or signaling modes of action. Structural PA is a precursor of glycerophospholipids (1). In mammals, structural PA is synthesized through two successive acylation reactions and can then be converted by phosphatidic acid phosphohydrolase to diacylglycerol (DAG), which is an important source for the biosynthesis of triacylglycerols and phospholipids (2). Three alternative biosynthetic pathways produce signaling PA (2). The first involves phospholipase D (PLD), which catalyzes the hydrolysis of the distal phosphodiester bond in phosphatidylcholine to form PA and choline. Phosphorylation of diacylglycerol by DAG kinase (DGK) constitutes the second pathway to produce PA and acylation of lysoPA by lysoPA-acyltransferases (LPAAT) the third. The complexity and diversity of the PLD, DGK and LPAAT families suggest that they are involved in specific cellular functions that

are probably not redundant. Signaling PA generated by these three enzymes has been implicated in cytoskeleton organization, cell survival and proliferation, membrane and vesicle trafficking (3). Yet, since PA is implicated, either directly or indirectly, in the biosynthesis of most phospholipids and triacylglycerols, its potential signaling functions may not be mutually exclusive from its intermediate biosynthetic role.

At the molecular level, PA has been found to interact with or regulate at least 50 different partners present in all organisms from yeasts and plants to mammals (4). These include GTPases, kinases, phosphatases, nucleotide-binding proteins and phospholipases (4). PA is a negatively charged phospholipid with a very small head group, which imposes a cone-shaped structure that can potentially affect membrane curvature (5,6). Thus, the search for a PA-binding motif among PA effectors is hampered by the fact that it is not always clear whether a given protein has specific affinity for PA or just an overall affinity for a negatively charged membrane topology. Hence, analyses of PA binding-polypeptide fragments have led to the idea that the only feature related to PA binding is a short stretch of positively charged residues (4). The actual view is that PA effectors do not contain a well-defined PA-recognition structural motif, but use either positively charged or surface-exposed hydrophobic residues, or a combination of both for binding to PA.

In analogy with other lipid signaling reactions, the interaction of proteins with PA often triggers their translocation from the cytosol to a specific membrane sub-compartment. However, PA can also directly regulate the activity of enzymes and trans-membrane proteins (7). For example, reconstitution studies have shown that PA inhibits the activity of the sarcoplasmic reticulum ATPase (8) and modulates the gating kinetics of potassium channels (9, 10). PA can also regulate small GTPases by modulating their regulatory proteins, guanine nucleotide

exchange factors (GEFs) and GTPase activating proteins (GAPs) (11). In terms of localization as well as function, PA effectors are found in almost all cellular compartments and are associated with a variety of cellular functions, although many of them regulate actin dynamics and membrane trafficking (3, 4, 7).

To date, the number of PA-specific probes that can be employed to gain insight into the localization and function of PA-binding proteins and/or image dynamics of PA pools in living cells is rather limited. Probes derived from the PA-binding domains (PABD) of the yeast protein Spo20p and Raf1 have been the most widely used. When expressed in cells, PABDs often localize to different subcellular compartments. For instance, the PABD of Spo20p (Spo20p-PABD) has been mostly described in the nucleus and plasma membrane in mammalian cells (6, 12), whereas the PABD of the yeast protein Opi1p (Opi1p-PABD) is reported to shuttle between the endoplasmic reticulum (ER) and the nucleus (13) and the PABD of the mammalian protein PDE4A1 (PDE4A1-PABD) is often found associated with the Golgi apparatus (14). Although it cannot be excluded that these different localizations result from cell specific expression, it may also suggest that PA probes are able to sense different pools of PA in a specific environment (15). To probe this idea, we performed an *in vitro* characterization and comparison of the Spo20p, Opi1p and PDE4A1 PA sensors using a novel semi-quantitative assay. Using macrophages as a cell model, we then investigated the intracellular distribution of the three PABDs and quantified the corresponding PA levels by mass spectrometry analysis. Macrophages internalize and eliminate pathogens and cellular debris using a membrane trafficking process known as phagocytosis. A number of intracellular organelles, including endosomes (16), lysosomes (17), ER (18, 19), and Golgi (20) have been reported to contribute to the formation of

the phagosome membrane during particle engulfment. At the molecular level, PLD has been described to be necessary for the formation and maturation of phagosomes along with efficient Fcγ receptor-mediated phagocytosis and has been identified on phagocytic pseudopods that engulf particles (21-24). A comparison of unstimulated macrophages with those undergoing frustrated phagocytosis, revealed that the three PABDs are useful tools to localize and distinguish variations in PA levels and species in subcellular compartments.

RESULTS

PABDs of Spo20p, Opi1p and PDE4A1 preferentially bind to PA – In the attempt to characterize and compare biosensors for PA, we prepared GST-fusion proteins composed of the PABD of Spo20p (fragment 50-91), Opi1p (fragment 103-191) and PDE4A1 (fragment 1-40). These PABDs were expressed in *E. coli* as GST-fusion proteins, purified and their interaction with PA was probed using a phospholipid overlay assay. The three PABDs displayed a strong preference for PA over other anionic lipids, at least in a non-membranous environment (Fig. 1A). As well, experiments performed with phospholipids inserted in a membrane using liposome flotation confirmed that the three sensors retained their preference for PA over PI(4,5)P₂ (Fig. 1B) and PS, another anionic phospholipid (data not shown).

In order to monitor PA binding of the three GST-PABDs in a lipid environment, we developed a semi-quantitative *in vitro* assay using NBD-labeled liposomes. As shown by confocal microscopy analysis (Fig. 1C), PA-containing fluorescent liposomes specifically bound to GST-Spo20p-PABD linked to GSH-sepharose beads, but not to control GST linked to GSH-sepharose beads. Based on this observation, the

binding of fluorescent liposomes (5% PE-NBD, 85% PC, 10% PA or PI(4,5)P₂) to GST-PABDs linked to GSH-sepharose beads was monitored by fluorimetry. Although significant binding of PI(4,5)P₂ containing liposomes to the three PABDs was detected, our observations clearly indicated a preferential binding of the PA liposomes over the PI(4,5)P₂ liposomes in all cases (Fig. 1D). When comparing PA binding to the three PABDs, it appears that Spo20p-PABD is by far the most potent PA sensor (Fig. 1D). The binding of the PA-containing liposomes to the PABDs was greatly reduced by specific point mutations in the PABD affecting either the α-helical conformation (Spo20p-PABD) or the positive charge clusters (Opi1p-PABD, PDE4A1-PABD) (Fig. 2A), indicating that the interaction depended partially on electrostatic interaction, but also on the structural conformation of the PABD.

PABDs of Spo20p, Opi1p and PDE4A1 are sensitive to the local lipid environment and to PA species – In a second series of experiments, the influence of the lipid composition was examined using fluorescent liposomes constituted of PC with variable degrees of saturation and a polyunsaturated form of PA (16:0/22:6). Two types of liposomes were used: liposomes made with unsaturated PC (dioleoyl-PC - DOPC) and liposomes made with PC having one saturated fatty acyl chain (palmitoyl-oleoyl-PC - POPC). As shown in Fig. 2B, the two types of liposomes bound preferentially Spo20p-PABD compared to Opi1p-PABD and PDE4A1-PABD. However, binding to Opi1p-PABD- and PDE4A1-PABD was sensitive to the PC composition of the liposomes. POPC/PA and DOPC/PA liposomes bound equally well Spo20p-PABD, but POPC liposomes were much less effective than DOPC liposomes in binding to Opi1p-PABD and PDE4A1-PABD (Fig. 2B), suggesting that PA binding capacity of Opi1p-PABD and PDE4A1-PABD were sensitive to the PC

nature present in liposomes. Unsaturated fatty acyl chains have a conical shape that creates lipid-packing defects in membranes (35). Together these observations suggest that Spo20p-PABD displays a higher affinity for PA compared to Opi1p-PABD and PDE4A1-PABD. On the other hand, Opi1p-PABD and PDE4A1-PABD are able to sense PA and changes in its lipid environment in the membrane, which is less the case for Spo20p.

We then examined the PA-binding specificity of the PABDs by using liposomes containing PA species with different fatty acid chains varying in length and saturation. All three PABDs showed an overall preference for long and unsaturated fatty acid chains, with Spo20p-PABD displaying in general a higher affinity (Fig. 3). However, differences in the relative PA preferences were noted. Mono- and bis-unsaturated PAs in *sn*2 position appeared to bind preferentially to the Spo20p-PABD and PDE4A1-PABD, but not such preference could be seen for Opi1p-PABD (Fig. 3). Of note, on the contrary to Opi1p-PABD, Spo20p-PABD and PDE4A1-PABD displayed reduced binding of polyunsaturated PA (Fig. 3). Increasing the *sn*1 chain length from 16 to 18 carbons increased the binding to Spo20p-PABD, whereas it reduced the binding to Opi1p-PABD and PDE4A1-PABD. PA(18:0/18:1) displayed the most potent binding to Spo20p-PABD, while PA(16:0/18:1) was preferred by PDE4A1-PABD (Fig. 3). The influence of the acyl chain length on PA binding was tested using bi-saturated PA because of limited commercial availability of these PA species (Fig. 3D). The shortest PA tested, PA(8:0/8:0), bound equally well to all three probes, but as the length of the acyl chain increased, Opi1p-PABD and PDE4A1-PABD bound less, while Spo20p-PABD bound more (Fig. 3D), suggesting that the probes differed in specificity regarding the acyl chain length of PA. Altogether, these observations suggest that the three PA sensors display a

specific PA preference profile in terms of length and saturation of the acyl chains and may be useful for sensing distinct PA species within membranes.

Effect of membrane topology and environment on the PA binding capacity of the PABDs of Spo20p, Opi1p and PDE4A1 – Differences in membrane curvature may also modulate the availability of PA molecules for interacting with target proteins and this can be reproduced by varying the size of liposomes. Among the different PA species, we decided to probe the effect of membrane curvature using liposomes containing mono-unsaturated PA (PA 18:0/18:1 – Fig. 4A) or bis/poly-unsaturated PA (PA 18:2/18:2 or PA 16:0/22:6 – data not shown). As illustrated in Fig. 4A, increasing the liposome radius from 50 nm to 1 μ m slightly reduced binding to the three PA probes. However, the effect of membrane curvature was not significantly different among liposomes composed of different PA species (data not shown). This observation is in contrast to the high sensitivity to curvature of the amphipathic lipid packing sensor (ALPS) motif of a Golgi tether that display almost no charge but a well-developed hydrophobic face (36).

As cholesterol has a critical influence on lipid packing, the effect of increasing the cholesterol concentration in PA-containing-liposomes was investigated. Cholesterol was found to increase the binding of liposomes to Spo20p-PABD and to a lesser extent to PDE4A1-PABD, but it reduced the binding to Opi1p-PABD (Fig. 4B). Similar results were obtained with DOPC and POPC liposomes (data not shown). Thus, the three PA probes are sensitive to the presence of cholesterol in the PA lipid environment.

In liposome reconstitution assays, the effect of electrostatics can be very pronounced. When binding occurs through a cryptic polybasic region, which like Velcro binds firmly above a threshold of negative charges, additional charges

provided by other lipids may contribute to the binding. To probe the effect of the net charge on the binding capacity of the PABDs, increasing amounts of phosphatidylserine (PS) were added to the liposomes. As the charge was increased, PA containing-liposome binding to Spo20p-PABD and PDE4A1-PABD was gradually enhanced, but there was no modification in the binding to Opi1p-PABD (Fig. 4C). Finally, we examined the effect of calcium, which can electrostatically interact with the two dissociable protons of the PA phosphate moiety, and hence modulate the number of charges available for interaction (39, 40). In the presence of 20 μ M calcium, PA-liposome binding to Spo20p-PABD and PDE4A1-PABD was increased, while binding to Opi1p-PABD appeared insensitive to this calcium concentration (Fig. 4D), again arguing for calcium sensitivity differences among the PA sensors.

The PABDs of Spo20p, Opi1p and PDE4A1 display specific intracellular localizations in macrophages - To promote particle engulfment, phagocytic cells remodel their plasma membrane and increase their surface area by incorporating intracellular vesicles and this process appears to require PLD-generated PA (18, 19). To investigate the intracellular sites of PA synthesis in the course of phagocytosis, we expressed the PABD probes fused to GFP (GFP-PABDs) in RAW 264.7 macrophages. In non-stimulated cells, GFP-Spo20p-PABD was largely concentrated in the nucleus, although to some extent it also underlined the plasma membrane, as indicated by partial co-localization with the isolectin IB4 (Fig. 5A), in agreement with the peripheral distribution observed previously (15). Like GFP-Spo20p-PABD, GFP-Opi1p-PABD was present in the nucleus, but also in the cytoplasm where it appeared associated with faint reticular structures, which partially co-localized with the ER marker

calnexin (Fig. 5A), arguing that Opi1p-PABD may bind PA in ER membranes of macrophages. In contrast, the distribution of GFP-PDE4A1-PABD was markedly different as it accumulated mostly in the perinuclear region and co-localized with giantin, a membrane protein of the Golgi apparatus (Fig. 5A). Of note, the three GFP-PABD proteins, in which PA binding was abolished by point mutations, were essentially distributed throughout the cytoplasm or the nucleus and apparently no longer membrane bound (Fig. 5B). Thus, when expressed in macrophages, the three PA probes are also able to distinguish between PA pools present in different subcellular compartments, suggesting that they most likely sense either different PA species and/or PAs in specific lipid or charge environments.

To examine the distribution of the GFP-PABDs during phagocytosis, we used the well-characterized frustrated phagocytosis assay. Particle phagocytosis can be mimicked in macrophages plated on BSA-anti-BSA IgG coated dishes and stimulated by a temperature shift from 4°C to 37°C, as shown in the images obtained by scanning electron microscopy (Fig. 6A). GFP-PABDs distribution in macrophages plated on BSA-anti-BSA IgG coated dishes at 4°C was similar to that of macrophages grown on glass coverslips (data not shown). In macrophages expressing GFP-Spo20p-PABD, frustrated phagocytosis resulted in a massive recruitment of the probe at the plasma membrane, especially on vesicular podosome-like structures, which were evident in basal sections of cells, concomitant with a net reduction of the nuclear localization of the probe (Fig. 6B-C). These results are in line with our previous observation using another PA probe GFP-Raf-PABD, which is also recruited to forming phagosomes during the phagocytosis of IgG-coated particles by RAW 264.7 macrophages (19). Interestingly, in phagocytosing macrophages, GFP-Opi1p-PABD was also observed at

the cell periphery and reduced in the ER (Fig. 6B arrows and Fig. 6C). On the other hand, frustrated phagocytosis did not alter the localization of GFP-PDE4A1-PABD on giantin-positive perinuclear structures (Fig 6B). Altogether, these data reveal major changes in PA levels and/or composition in the plasma membrane, but also in the ER of macrophages undergoing phagocytosis.

Lipidomic analysis confirm subcellular changes in PA levels detected by the PABD probes during frustrated phagocytosis – To confirm the data obtained using the PA sensors, cellular and subcellular PA levels in macrophages before and after induction of frustrated phagocytosis were analyzed by mass spectrometry. Fractions enriched in cellular organelles from non-stimulated and stimulated macrophages were obtained by differential centrifugation of cell lysates (Fig. 7A). In cell lysates, the total PA corresponded to approximately 2000 pmol/mg protein, which is in line with a previous lipidomic profiling of this cell line (32). After 30 min of phagocytosis, the total cellular level of PA increased by $39.6 \pm 4.3 \%$ (Fig. 7B). While no significant changes were observed in the cytosol, mitochondrial and nuclear fractions, strong differences in PA levels were detected in the ER and plasma membrane containing fractions (Fig. 7B). PA levels doubled in the plasma membrane fraction, and inversely, decreased by 50% in the ER fraction. These data are in line with the changes in localization observed with the PA sensors, GFP-Opi1p-PABD and GFP-Spo20p-PABD. In macrophages, phagocytosis is accompanied by a strong increase in PA at the plasma membrane, in line with the presence of PLD at the cell periphery (18, 19), and a concomitant reduction in PA levels in the ER, an observation that may support the possible, up to now largely debated, contribution of the ER to phagocytosis (15). A detailed MS analysis of the ER membranes

fractions allowed us to detect significant levels of 44 different PA species (data not shown). Intriguingly, at the exception of four poly-unsaturated omega-3 forms of PA, for which levels were moderately reduced after frustrated phagocytosis, the levels of each species was very similarly reduced by nearly 55% (data not shown).

PABD probes binding does not depend on proteins present in membrane compartments – The binding of GST-Spo20p-PABD- and GST-Opi1p-PABD to plasma or ER enriched membrane fractions (see Fig. 7B for purity estimation) was estimated by measuring unsaturated fatty acid phospholipid levels associated to GST-PABDs linked to GSH-sepharose beads. No significant lipid binding was found when plasma or ER enriched membranes were incubated with control GST linked to GSH-sepharose beads (Fig. 8A). GST-Spo20p-PABD preferentially bound to plasma membranes compared to ER membranes whereas GST-Opi1p-PABD preferentially bound ER membranes (Fig. 8A). This finding is in line with the intracellular distribution of GFP-Spo20p-PABD and GFP-Opi1p-PABD observed in macrophages (Fig. 5). To probe whether the binding preferences of the PABD probes might involve interactions with membrane-associated proteins, plasma and ER membrane fractions were treated with either trypsin or proteinase K to hydrolyze associated proteins (Fig. 8 inset) and incubated with GST-Spo20p-PABD or GST-Opi1p-PABD. Both protease treatments did not significantly change the amount of membrane bound to the PA sensor beads (Fig. 8B, C), indicating that membrane-associated proteins do not contribute to the binding capacity and selectivity of the PA sensors. In other words, the PABD probes most likely sense the PA species and their lipid environment within membranes.

DISCUSSION

Relatively limited information is available to model the interaction between PA and PA-binding proteins. In the original study describing the ability of Spo20p to bind specifically PA, the PA-binding domain was predicted to form an amphipathic helix with both positively charged and hydrophobic amino acid residues contributing to PA binding (12). In agreement with these secondary structure predictions, we recently found that the PA-binding region of Spo20p is largely formed by an amphipathic helix spanning from G56 to L78 followed by a smaller helix on the C-terminal part (F84 to K89) (41). Using molecular dynamic simulation, the Spo20p PA-binding polypeptide was predicted to adopt an interfacial orientation with most of the hydrophobic residues embedded in the membrane, while positively charged residues coordinate several PA head-groups (41). Bioinformatics predicts that the three PA sensors tested here adopt amphipathic helix conformations with four to six charged residues sequestered on one side of the helix, whereas the other side of the helix is enriched (five to seven) in hydrophobic residues (Fig. 9A). Most likely, PA binding by these short polypeptides does not involve a core-structured motif, but hydrophobic residue insertion into lipid bilayers and direct ionic interaction between basic residues and several molecules of PA. Spo20p-PABD is clearly the probe that binds the most efficiently PA. This may reflect a structure that is more optimal for PA binding in Spo20p-PABD and could involve a higher density of hydrophobic and charged residues on each side of the α -helix (Fig. 9A). Spo20p-PABD also contains a unique histidine-rich polar face that may contribute to some of its specific characteristics for binding to PA in membranes. By changing the liposome amounts in the binding assay, we were able to calculate the concentration of

liposomes required for half maximal binding. From these values the apparent K_d for PA(18:0-18:1) binding by GST-Spo20p-PABD, GST-PDE4A1-PABD, and GST-Opi1p-PABD was respectively of 2.2 μ M, 6.8 μ M, and 4.5 μ M, respectively. The low μ M range for each of PABD is reflecting as expected a low affinity interaction. These apparent K_d s are, however, rather crude estimation of the affinity since both the liposomes and the PABD should be considered as soft matters. Indeed it is likely that the PABDs adopt an unstructured conformation in solution and progressively organize in α -helical structures as they associate with liposome membranes. Furthermore it should be noted that it is likely that the different PABDs bind to more than one PA molecule.

The present *in vitro* observations indicate that the three PA sensors tested here require a specific membrane environment for optimal PA binding (summarized in Fig. 9B). Our findings are in agreement with the observations showing that different PA binding proteins display differential binding as a function of membrane curvature stress and chemical properties (45). Although it is impossible to completely rule out that additional factors, such as transmembrane proteins, contribute to the distinct subcellular distribution found in macrophages, the *in vitro* data suggest that the probes are able to distinguish not only the level but also the species of PA in a particular lipid environment. In agreement, the binding preference of GST-Spo20p-PABD and GST-Opi1p-PABD towards either plasma or ER membranes remains unchanged even after effective proteolysis (Fig. 8). Importantly, preferential binding for a particular PA was observed only when PAs were included in a lipid environment. When the GST-PABD probes bound to GSH-sepharose-beads were incubated with a mixture of egg or soybean PA of known composition, mass spectrometry analysis

showed that the proportion of each of the PA species bound to the sensors was strictly similar to the original PA mixtures (data not shown). This clearly indicates that the PABD-PA interaction involves parameters occurring in a membrane context. Thus, the three probes studied here present their own spectrum of binding preferences to different PA species based on fatty acid chain length and saturation. Although the subcellular distribution of PA sensors might rely on additional parameters such as the luminal pH (46), it is noteworthy that the change of subcellular distribution observed here during frustrated phagocytosis was correlated with a change in PA level in the corresponding membrane compartments. Furthermore the fact that point mutations that reduce charged clusters (Opilp-PABD and PDE4A1-PABD) or the α -helical conformation (Spo20p-PABD) strongly reduce binding of PA containing liposomes and induce membrane to cytosol relocation supports the notion that the intracellular distribution of these probe is highly sensitive to PA levels in membranes. These PABD probes might therefore represent useful tools to detect the PA level and composition in distinct subcellular membranes and the possible variations during cell activation.

Binding of Spo20p-PABD to PA is favored by high charges, cholesterol and calcium, and at a lesser extent by the length and saturation of the fatty acyl chains and packing defects (Fig. 9B). These characteristics are typical of endosomal and plasma membranes (37). In fact Spo20p is the yeast homologue of SNAP-25 necessary for spore formation. As shown here, when expressed in macrophages (summarized in Fig. 9C), GFP-Spo20p-PABD is recruited to the plasma membrane during phagocytosis similar to what is observed in stimulated PC12 cells undergoing neurosecretion (6, 15). Altogether, our findings suggest that GFP-Spo20p-PABD might represent a PA sensor particularly suitable to detect PA

modifications in the plasma membrane and derived organelles. It is noteworthy to mention that a probe containing two Spo20p-PABD fused to GFP or GST through two coiled-coil motifs displayed similar characteristics regarding binding to PA, although it also bound avidly to PI(4,5)P₂ (43). Increased binding to PI(4,5)P₂ of this double-Spo20p-coiled-coil, potentially a consequence of the apposition of tandem Spo20p motives, reduces the cellular interest of this tool as a specific PA sensor. This construct has, in consequence, not been used as widely as GFP-Spo20p-PABD for monitoring PA in cells (2, 6, 15).

The biophysical membrane parameters for optimal binding of Opilp-PABD are high packing defects, short and unsaturated PA, low cholesterol levels and little charge, which all are hallmarks of the ER membrane (35), where this sensor accumulates in macrophages (summarized in Fig. 9C). Of note this is only in partial agreement with the findings of Hofbauer et al., (2014), where the full-length Opilp protein was found to preferentially bind to short, but saturated PA (43). These differences may result from additional domains/interactions occurring in the full-length Opilp, but also from the limited number of PA species used in their liposome flotation assay (44); the latter being clearly less quantitative than the fluorimetric assay presented here. In agreement with our findings, the lipidomic analysis revealed that saturated PA species are in extremely limited amounts in the ER fraction purified from cultured macrophages (data not shown), making it unlikely that these PA species played a role in recruiting GFP-Opilp-PABD to the ER in macrophages. In yeast Opilp is a transcriptional repressor, which is normally localized on the ER via its interaction with PA. Under conditions where the biosynthetic PA pool is depleted, Opilp-PABD translocates to the nucleus where it represses genes involved in inositol synthesis (13). Thus, the GFP-Opilp-

PABD sensor may be ideally suited to follow PA dynamics in the ER of most cellular models.

The PDE4A1-PABD appeared somehow intermediate in these requirements and was found to associate with the Golgi, which makes the transition between the lipid-packing defects and electrostatics territories (3). In agreement, the PABD of PDE4A1 has previously been shown to consist of two α -helices linked by a hinge; the two-tryptophan residues of the second helix are important for lipid insertion, while the first helix is important for a calcium-sensitive association with the Golgi (47).

Finally it is also worth mentioning that the PA binding signals of Spo20p and Opilp overlap with nuclear targeting signals, suggesting some functional coupling between nuclear traffic and PA recognition. On one hand, specific protein-PA interactions may occur in the nucleus, but on the other hand regulating extra-nuclear PA levels may represent a physiological control mechanism for the localization of these proteins, as it has been suggested for Opilp (13). Moreover, PA binding near the PX domain of p47^{phox} has been reported to regulate its affinity for PI(3,4)P₂ (48), indicating that PA binding can also indirectly affect membrane affinity by modulating additional membrane interaction domains. Thus, in some circumstances membrane anchorage may require more than one binding site or lipid modification to facilitate cytosolic translocation to membranes in living cells. The precise characterization of other PABDs described to date, will undoubtedly prove helpful in understanding the molecular mechanisms of protein-PA interactions and generate novel PA sensors that are critically needed to follow PA dynamics in the cytosolic leaflets of cellular membranes in real time and ultimately gain insight into the complex structural and signaling functions of the simplest glycerophospholipid.

The biosynthesis and consumption of PA are regulated in cells by several different enzymatic routes (2). Biosynthetic pools of PA are mainly formed in the ER by the assembly of glycerol 3-phosphate and fatty acyl CoA and are an essential intermediate for the formation of other phospholipids or phosphoinositides. Signaling and more directly functional pools of PA are formed from PC via the action of PLD and by the phosphorylation of DAG following, for example, hydrolysis of PI(4,5)P₂ on distinct subcellular membrane compartments. The present finding that PA levels are dramatically modified in the plasma membrane and in the ER during phagocytosis, as revealed by mass spectrometry analysis and the use of the GFP-PABD sensors, argues that signaling PA might be produced in both the plasma membrane and ER. It is worthwhile mentioning that fluctuations in PA levels in both of these compartments have been observed following lipopolysaccharide activation of macrophages, although in this case the PA level decreased in the plasma membrane fraction and increased in the ER (34). During frustrated phagocytosis, the marked increase in PA levels in the plasma membrane fraction and the recruitment of the Spo20p-PABD and Opilp-PABD probes at the plasma membrane is in line with previous observations showing that PA synthesis by PLD1 and PLD2 positively contributes to Fc γ R-mediated phagocytosis (21-24). In the present case, the exaggerated phagocytic response provoked in the frustrated phagocytosis model probably requires a more extensive remodeling of the plasma membrane and a greater contribution from the intracellular membrane sources. This may explain why nearly half of PA has disappeared from the ER during frustrated phagocytosis. One possible explanation for this finding is that PA has been converted to other signaling lipids in the ER as a consequence of phospholipase A or PA-phosphatase activity. Alternatively, there may have

been a direct transfer of PA to the plasma membrane at ER-plasma membrane contact sites (49). In line with this hypothesis, we found that the levels of most of the 44 different species detected in our MS analysis decreased proportionally after frustrated phagocytosis (data not shown). This data could suggest an en bloc transfer of PA from the ER to the plasma membrane during frustrated phagocytosis. Although appealing, this later possibility needs to be directly investigated and the molecular mechanisms of PA transfer remain to be identified. However, in support of this possibility, extended synaptotagmins which are ER-resident integral membrane proteins have recently been reported to serve as calcium-regulated tethers between the ER and the plasma membrane, thereby providing a molecular support for lipid transfer between ER and the plasma membrane in receptor-stimulated cells (50). In macrophages, the contribution of the ER to phagocytosis has been largely debated and remains controversial (18, 19). Yet, ER proteins appeared enriched in phagosomes (19) and additional work has suggested that ER contribution to phagocytosis occurs preferentially upon ingestion of large particles that requires important membrane addition to the plasma membrane (51). Thus the present results support the idea that the ER might contribute to the increased PA levels at the plasma membrane during phagocytosis.

MATERIAL & METHODS

Antibodies – Anti-Histone H3, anti-Giantin (Ab24586), anti- Na^+/K^+ -ATPase (Ab58475), anti-HSP-60 (Ab46798), and anti-Calnexin (Ab22595) were purchased from Abcam, anti-CD64 (MAB2074) from R&D Systems, anti-GST (G1160) from Sigma, and anti-GAPDH (2118S) from Ozyme.

Expression of recombinant GST-PABD proteins – Fragments containing amino acids 50-101 from Spo20p (GenBank DAA09915.1) (12), 102-191 from Opi1p (GenBank M57383.1) (13), and 1-40 from PDE4A1 (GenBank M26715) (14) were used as PABD. The Spo20p-PABD was amplified by PCR from the pEGFP-C1-PABD-Spo20p construct (6) using the forward primer 5'-CGGGATCCCTCGAGCGTCTAGAATGG-3' and reverse primer 5'-GCGAATTCTTAAGTAGTCTTAGTGGCGTC-3', as previously described. The Opi1p-PABD was amplified by RT-PCR from yeast DNA (gift from Dr. S. Friant, Université de Strasbourg, France) using the forward primer 5'-AGTGGATCCGATGAGTTCTTCACCAAC-3' and reverse primer 5'-AGTGAATTCCGATGTCTCGTCCTCGCC-3'. The PDE4A1-PABD corresponding to the N-terminal regulatory domain of a cAMP-specific phosphodiesterase (PDE) was amplified by RT-PCR from rat cDNA as described (14), using the forward primer 5'-AGCGGATCCATGCCTCTGGTTGAC TTC-3' and reverse primer 5'-AGCGAATTCTGACCTGCTCATTTC GAC-3'. The PABDs were inserted in frame into pGEX4T1 as previously described (25). Point mutations were created to abolish PA binding on Spo20p-PABD(L67P), Opi1p-PABD(K126M-R127G), and PDE4A1-PABD(K25A-R26A) with a Quick-Change site-directed mutagenesis kit from Stratagene (26). Mutations and sequences of the clones were verified by automated sequencing. Large-scale production of chimeric GST-PABD proteins has been previously described (27). Briefly, expression was induced at 37°C and fusion proteins were purified on glutathione (GSH)-Sepharose (28) and purity was estimated to be >95% by Coomassie blue staining of SDS-PAGE gels. The quantity of purified protein was determined from this dye-binding assay using bovine serum albumin as a standard (29). Protein aliquots were stored at -80°C.

Lipid overlay assay – Following the protocol indicated by Molecular Probes, nitrocellulose membrane strips containing various phospholipids were saturated with blocking solution (phosphate buffered saline containing 3% bovine serum albumin and 0.1% Tween 20 (PBS-BSA-Tween)) for 4 hr at 4°C. Then the strips were incubated for 2 hr at 4°C with GST-PABD-proteins (diluted to 0.01 µg/mL in PBS-BSA-Tween). GST alone was used as a control. Subsequently, the strips were analyzed by Western blot using an anti-GST antibody (Molecular Probes) and revealed by chemiluminescence. Images were acquired with a Chemismart 5000 (Vilber Lourmat) and quantified using Image J software.

Liposome flotation and binding assays -

Liposome preparation was performed as previously described (30). Lipids solubilized in chloroform were purchased from Avanti Polar Lipids. Liposome mixtures were prepared in mass ratios composed of 85% phosphatidylcholine (PC), 5% NBD-labeled phosphatidylethanolamine (PE-NBD), and 10% PA, PC, PE, phosphatidylserine (PS), or phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) or with other molar ratios as indicated. Lipids were dried in a stream of nitrogen gas and kept under vacuum for at least 2 hrs. Dried lipids were then suspended at a concentration of 1.65 mg/mL in liposome-binding buffer (LBB; 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM MgCl₂) by three freeze and thaw cycle and were extruded using a Mini-Extruder (Avanti) through polycarbonate track-etched membrane filters to produce liposomes usually 200-nm diameter, unless otherwise indicated. The size distribution of liposomes was estimated by dynamic light scattering using a Zetasizer NanoS from Malvern Instruments equipped with a 4 mW laser. Samples were diluted 1:100 in LBB buffer and analyzed at 20°C using 10 runs, each comprised of 14 measurements.

To determine the association of the GST-PABD constructs with these liposomes, a liposome flotation assay was adapted from the protocol described by Bigay *et al.* (31). Briefly, 5 µg of each probe was incubated with 150 µL of NBD-labeled liposomes (1 mg/mL) or LBB at room temperature for 15 min. This suspension was mixed with 100 µL of LBB containing ice-cold 75% sucrose, resulting in a 30% sucrose layer, which was overlaid with 200 µL of 25% sucrose solution prepared in ice-cold LBB and 150 µL of ice-cold LBB. The step gradients were centrifuged at 200,000 × *g* for 1 hr at 4°C in a Beckman swinging rotor (SW-32 Ti). The top 150 µL (T), middle 200 µL (M), and bottom 250 µL (B) layers were manually collected from the top and analyzed for fluorescence at excitation and emission wavelengths of 485 and 535 nm respectively with Mithras (Berthold) Fluorimeter, to quantify the percentage of liposomes present in each fraction. Equal volumes of fractions were run on SDS-PAGE and immunoblotted with an anti-GST antibody as described previously (32).

A second approach was carried out to quantify liposome binding to GST-PABDs linked to GSH-sepharose-beads. GST and GST-PABDs (330 pmol) bound to GSH beads were washed once with 1 mL LBB medium before being incubated for 20 min in the dark at room temperature and under agitation with liposomes containing a 10-fold molar excess of PA relative to the quantity of GST-proteins in a final volume of 200 µL LBB. Beads were washed three times with 1 mL ice-cold LBB buffer and collected by centrifugation at 3000 rpm 5 min. Liposome binding to the PABDs was estimated by measuring the fluorescence at 535 nm with a Mithras (Berthold) Fluorimeter. Triplicate measurements were performed for each condition. Fluorescence measured with GST linked to GSH-sepharose-beads alone was between 3 and 4 A.U. and was subtracted from sample measurements.

Membrane binding assay and saturated lipid quantification – GST and GST-PABDs (330 pmol) bound to GSH beads were washed once with 1 mL LBB medium before being incubated for 30 min at room temperature and under agitation in LBB with plasma and ER membranes (containing ~400 µg of unsaturated lipids), purified as described below. Beads were washed once with 1 mL ice-cold LBB buffer and collected by centrifugation at 5000 rpm 1 min. Lipid binding to the PABDs was estimated by measuring the amount of unsaturated lipids using a colorimetric lipid quantification kit (Clinisciences, France) as described by the manufacturer. Briefly standards and samples were incubated with 150 µL of sulfuric acid and heated at 90°C for 10 min, before incubation with vanillin at 37°C for 15 min. The absorbance at 540 nm was measured with a Mithras (Berthold) Fluorimeter. Samples of membrane fractions containing ~200 µg of protein were incubated without or with trypsin at 0.1 µg/µL or proteinase K at 0.2 µg/µL in 20 µL for 2 hrs at 37 °C. Samples were incubated for 1 hr at 75°C for protease heat-inactivation before incubating samples with GST-PABDs bound to GSH beads.

GFP-PABD probes BD was described previously (6). GFP-Opilp-PABD and GFP-PDE4A1-PABD were generated by inserting the PABDs in *Bgl*II and *Eco*RI digested pEGFP-C1. Mouse macrophage RAW 264.7 cells were maintained in RPMI-Glutamax medium (GIBCO, Thermo Scientific), supplemented with 10% heat-inactivated foetal calf serum, 10 mM HEPES, 1 mM Na pyruvate, 50 µM β-mercaptoethanol, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C under 5% CO₂. For frustrated phagocytosis studies cells were plated on Upcell dishes (Nunc, Thermo Scientific) to facilitate the preparation of cell suspensions and grown in macrophage serum-free medium (Thermo Scientific). RAW 264.7 cells at

50% confluence on glass coverslips or in 6-well Upcell plates were transfected with GFP-PABD plasmids using Lipofectamine 2000 according to the manufacturer's protocol (Thermo Scientific).

Frustrated Phagocytosis - Frustrated phagocytosis reflected by cell spreading on immobilized immune complexes was carried out either on Petri dishes (Falcon, 10 cm) for cellular fractionation or on glass coverslips for imaging. Surfaces were pre-coated with BSA (3%) and either rabbit anti-BSA IgG (0.04 mg/mL, Invitrogen A11133, Thermo Scientific) or sheep anti-BSA (0.04 mg/mL, Euromedex GTX77113) adapted from the protocol of Bainton *et al.* (20). Suspensions of RAW 264.7 cells (2×10^7 /10 cm dish for fractionation; $1-2 \times 10^5$ /12 mm coverslip or $1-2 \times 10^4$ /glass-bottom microwells (MatTek) for microscopy) in cold serum-free medium were then allowed to attach to pre-coated surfaces at 4°C for 1 hr. Cell spreading was initiated by warming to 37°C for 30 min for fractionation and 10, 20 or 30 min for microscopy. As controls, macrophages were either plated on surfaces pre-coated with immune complexes or with BSA alone and maintained at 4°C.

Immunolabeling and confocal microscopy - After washing in cold medium, adherent macrophages transfected with GFP-PABD constructs were fixed with 4% paraformaldehyde in serum free medium for 10 min at room temperature. Then, transfected cells were either directly labeled with Hoechst and Isolectin B4 (IB4-A647 Molecular Probes, Thermo Scientific) or permeabilized in PBS containing 4% paraformaldehyde and 0.1% Triton-X100, for 10 min at room temperature. Calnexin (1:200 dilution) and giantin (1:500 dilution) antibodies were used as markers for the ER and the Golgi apparatus respectively, and revealed with a goat-anti-rabbit IgG-Alexa 647 (1:1000 dilution, Molecular Probes, Thermo

Scientific). The cellular distributions of the PA probes and markers were examined with a Leica SP5 II confocal microscope equipped with an oil immersion 63x objective (Plan Apochromat n.a.=1.4). Digital images were acquired in the equatorial and basal planes. Mask images were generated as described previously (33).

Scanning electron microscopy - To verify frustrated phagocytosis, as measured by cell spreading on coated coverslips, macrophages were prepared for scanning electron microscopy. Phagocytosis was stopped by placing samples on ice and rinsing with cold macrophage serum free medium. Cells were then fixed with 2% glutaraldehyde in serum free medium for 30 min and a further 30 min with 2% glutaraldehyde in phosphate buffered saline, before being dehydrated and embedded in Durcupan. After decapping samples with propylene oxide, they were carbon-coated and examined with a Hitachi S800 scanning electron microscope.

Subcellular fractionation - 1.5×10^8 RAW 264.7 were harvested as described (34), by scraping in 40 ml of PBS, pelleted at 200 g for 7 min, resuspended in 40 mL of isolation medium (250 mM sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA), and pelleted again to remove salts (see Fig. 7A). For effective homogenization, cells were subjected to mild osmotic shock in 40 mL of slightly hypotonic medium (isolation medium containing 100 mM sucrose) and thereafter pelleted. The supernatant was set aside; the cell pellet was carefully transferred to a glass Dounce homogenizer, 10 mL of the supernatant was added, and the mixture homogenized by 40 strokes with a tight-fitting pestle. The resulting slurry was then combined with the remaining supernatant. The homogenate was brought to an isotonic state by the addition of 3.5 mL of the hypertonic medium (isolation medium containing 1.78 M sucrose) and then

supplemented with 2 mM $MgCl_2$ to preserve nuclei through subsequent steps. Differential centrifugation parameters were as follows: 200 g for 10 min to pellet nuclei/unbroken cells (crude “nuclear” pellet), 5,000 g for 10 min to pellet mitochondria, and 100,000 g for 1 hr to pellet microsomes and plasmalemma fragments. Postnuclear and post-mitochondrial supernatants were additionally purified by centrifugation at 300 g and 5,000 g for 10 min, respectively, to remove residual nuclei and mitochondria. The crude nuclear and mitochondrial pellets were then washed and pelleted in Mg^{2+} -containing and Mg^{2+} -free media, respectively. The supernatant from the 100,000 g spin was retained as the cytosolic fraction. The nuclear, mitochondrial, and microsomal pellets were subjected to centrifugation through OptiPrep step gradients in an SW-32 Ti rotor. Gradient media was prepared according to the manufacturer’s instructions using the isolation medium described above; for nuclear preparation, the medium was supplemented with 5 mM $MgCl_2$. To purify nuclei, the crude nuclear pellet was adjusted to 25% OptiPrep (final volume = 12 mL), and OptiPrep gradients were formed from the bottom up in three 12 mL tubes (4 mL 10%, 4 mL nuclei in 25%, 2.5 mL 30%, 1.5 mL 35% of Optiprep) and centrifuged at 10,000 g for 20 min. Nuclei were recovered at the 30/35% OptiPrep interface. The mitochondrial and microsomal pellets were resuspended in isolation medium adjusted to 35% OptiPrep (final volume = 6 mL) and fractionated by flotation for 2 hr at 50,000 g in three 12 mL tubes through a gradient was formed with 2 mL 10% OptiPrep, 4 mL 17.5% OptiPrep, 4 mL 25% OptiPrep, and 2 mL of the respective pellets resuspended in 35% OptiPrep. Mitochondria were collected at the 17.5/25% interface; plasmalemma and ER at the 10/17.5% and 17.5/25% interfaces, respectively. A third fraction, originating from the microsomal pellet, at the dense

25/35% interface, was termed heavy microsomes. All samples were frozen and stored at -80°C. Purity of fractions was verified by immunoblots using specific markers for the different fractions as described previously (33).

Lipidomic analysis - Total lipids from cell samples were extracted by the method of Bligh & Dyer (35). Extracts were mixed with chloroform:methanol (4:1), vortexed for 10 sec and left under agitation for 1 hr at 4°C. After a 5 min centrifugation at 13,000 rpm, the organic phase (150 µL) was recovered and analyzed by mass spectrometry (UPLC/MS/MS) on an Acquity UPLC system Waters corp. (Milford, USA) coupled to a Quattro Premier XE triple Quadrupole MS system Waters Micromass. The sample (3 µL) was injected into an Acquity UPLC BEH HILIC Amide precolumn (2.1 mm x 5 mm, 1.7 µm particle size), coupled to a Waters Acquity UPLC BEH Amide column (2.1 mm x 100 mm, 1.7 µm particle size) and maintained at 28°C. The column was eluted with a mix of acetonitrile 99.5%, ammonium hydroxide 0.5% (A) and acetonitrile 80%, water 19.5%, ammonium hydroxide 0.5% (B). The flow rate was 0.4 mL.min⁻¹ using the following elution protocol: 93% A for 2 min, followed by the gradient, 93% A to 60% A in 1 min, 60% A to 50 % A in 0.5 min, 50% A to 40 % A in 1.5 min. The composition of the mobile phase was then returned to initial conditions as follows: 40% A to 50% A in 2 min, 50% A to 80% A in 2.5 min, 80% A

to 93% A in 0.5 min and maintained in 93% A for 2 min. UV spectra were recorded from 200 to 500 nm. The system was run by Mass-Lynx software (version 4.0). The ESI source was used in positive and negative mode with a capillary voltage 3.4 kV; RF lens at 0 V, resolution (LM1, HM1, LM2, HM2) 15, ion energy 1 and 2:0.5. Source and desolvation temperatures were 135 and 400°C. Flows rates of nitrogen for nebulizer and desolvation were 50 and 900 L.h⁻¹. Pressure of the argon collision gas was 3.0 * 10⁻³ mbar. Full-scan, Selected Ion Recording and Daughter Scan mode were used for qualitative analyses. Quantitative PA analyses were made based on MS/MS Multiple Reaction Monitoring (MRM) as described (36). Briefly, MRM transitions for individual PAs were determined using PA standards (Avanti Polar Lipids, Alabaster, AL, USA). The PAs were identified as deprotonated parent ions [M-H]⁻, cone energy was optimised for each PA and set to 44 V. The predominant daughter fragment ions were then used for quantitative MRM analysis. After optimisation, the collision energy was set to 44V. MRM transitions and specific retention times were used to selectively monitor PA.

Statistical analysis - Number of experiments and repeats are indicated in figure legends. Normality of the data distribution was verified with ANOVA test and statistical analysis was performed with t-tests relative to the indicated control.

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ABBREVIATIONS

AU, Arbitrary Units; BSA, bovine serum albumin; DAG, diacylglycerol; DGK, diacylglycerol kinase; DOPC, dioleoyl phosphatidylcholine; DOPS, dioleoyl phosphatidylserine; ER, endoplasmic reticulum; GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GSH, glutathione; GST, glutathione-S-transferase; LBB, liposome-binding buffer, LPAAT, lysoPA-acyltransferase, MRM, multiple reaction monitoring; MS, mass spectrometry; NBD, *N*-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl); PA, phosphatidic acid; PABD, PA-binding domain; PBS, phosphate buffered saline; PC, phosphatidylcholine; PDE, phosphodiesterase; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI(3)P, phosphatidylinositol-3-phosphate; PI(3,4)P₂, phosphatidylinositol 3,4-bisphosphate; PI(3,5)P₂, phosphatidylinositol 3,5-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-triphosphate; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PI(5)P, phosphatidylinositol 5-phosphate; PLD, phospholipase D; POPC, palmitoyl-oleoyl phosphatidylcholine; PS, phosphatidylserine; SIP, sphingosine-1-phosphate.

The authors declare that they have no conflicts of interest with the contents of this article.

CONTRIBUTIONS

N.K., E.T., T.T., L.F., D.H., S.C.G., N.J.G., and N.V. performed and analyzed experiments. N.V. and N.J.G. designed the experiments. N.V., N.J.G., and M.F.B. wrote the manuscript. All authors revised the manuscript.

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FIGURES

Figure 1: Spo20p-PABD, Opi1p-PABD and PDE4A1-PABD preferentially bind to PA.

(A) Quantification of Spo20p-PABD, Opi1p-PABD and PDE4A1-PABD binding to phospholipids on nitrocellulose membrane strips (means $\pm$ SEM, n=4 independent experiments). The binding to GST alone was less than 0.1 A.U. and this background was subtracted from samples. A preference for PA (boxed) was observed for all of these probes. (B) Liposome flotation assay. To further validate PA specificity for Spo20p-PABD, Opi1p-PABD and PDE4A1-PABD, each GST-probe was incubated with LBB buffer containing fluorescent liposomes (5% PE-NBD, 85% DOPC and 10% PA, or PI(4,5)P₂) and separated on a sucrose density gradient by ultracentrifugation. Equal volumes of the top (T), middle (M), and bottom. More than 95% of the fluorescence was recovered in layers M and T indicating the presence of liposomes in these fractions. (B) layers were run on SDS-PAGE and immunoblotted with an anti-GST antibody (n=3 independent experiments). (C) Confocal images of GST and GST-Spo20p-PABD coupled to GSH-sepharose beads after incubation with liposomes (5% PE-NBD, 85% DOPC, 10% PA). Fluorescent liposomes accumulated on the surface of GST-Spo20p-PABD beads. Arrows indicate dots of fluorescence on the bottom or top of beads. Bar = 50 μ m. (D) Semi-quantitative fluorescent liposome assays with GST-PABD beads and liposomes (5% PE-NBD, 85% DOPC and 10% PA or PI(4,5)P₂). A 10-fold molar excess of PA or PI(4,5)P₂ relative to that of GST-PABD was used. Binding of liposomes to GST-PABD beads was estimated by measuring the total fluorescence and subtracting the background (see methods). Fluorescence measured when liposomes were incubated with GST alone was around 3 A.U. and was subtracted. Results are presented as means $\pm$ SD (triplicate measurements pooled from 3 experiments, * p>0.05, ** p>0.01, and *** p>0.001).

Figure 2: Effect of lipid packing defects on Spo20p-PABD, Opi1p-PABD and PDE4A1-PABD binding to PA.

(A) Semi-quantitative fluorescent liposome assays with wild-type GST-PABD beads or mutated Spo20-PABD-(PL67P) beads, Opi1p-PABD-(K126M-R127G) beads, PDE4A1-PABD-(K25A-R26A) beads and liposomes (5% PE-NBD, 85% DOPC, 10% PA). Results are presented as means $\pm$ SD (of triplicate measurements pooled from 3 experiments, * p>0.05, ** p>0.01, and *** p>0.001). (B) Semi-quantitative fluorescent liposome assays with GST-PABD beads and liposomes with variable packing defects. GST-PABD beads (330 pmol) bound to GSH beads were incubated with liposomes containing 5% PE-NBD, and 85% DOPC or POPC, in addition to 10% PA (16:0/22:06). Results are presented as means $\pm$ SD (of triplicate measurements from a representative experiment, n=3, ** p>0.01).

Figure 3: Effect of PA fatty acyl chain composition on PA binding of Spo20p-PABD, Opi1p-PABD, and PDE4A1-PABD.

(A-C) GST- and GST-PABD-beads were incubated with liposomes containing 5% NBD-PE, 85% of DOPC, and 10% of the indicated PA. Boxed PA species present the highest binding to PABDs and significant differences in binding are indicated (n=3 independent experiments, * p>0.05, ** p>0.01, and *** p>0.001). Data are presented as means $\pm$ SD (of triplicate measurements from a representative experiment, n=3). (D) Effect of increasing fatty acid chain lengths on saturated PA on binding to GST-PABD beads. Background fluorescence measured when liposomes were incubated with GST beads alone was subtracted. Data are presented as means $\pm$ SD (of triplicate measurements from a representative experiment, n=3). Significant differences from PA(8:0/8:0) containing liposomes are indicated (n=3 independent experiments, * p>0.05 and ** p>0.01).

Figure 4: Effect of membrane curvature, cholesterol, surface charge and calcium on PA binding of Spo20p-PABD, Opi1p-PABD and PDE4A1-PABD. (A) GST-PABD beads were incubated with liposomes of different sizes (50 nm, 200 nm, 1 μ m) containing 5% PE-NBD, 85% of DOPC, and 10% of PA (18:0/18:1). Results are presented as means $\pm$ SD. Significant differences to 200 nm liposomes are indicated (n=3 independent experiments, * $p > 0.05$). (B) GST and GST-PABD beads were incubated with liposomes containing 5% PE-NBD, 85% of DOPC, 10% PA(18:0/18:1). The % cholesterol represents the % that replaces DOPC in liposomes. Data are presented as means $\pm$ SD. Significant differences were observed between 0 and 30% cholesterol for each condition as indicated (n=3 independent experiments, * $p > 0.05$, ** $p > 0.01$ and *** $p > 0.001$). (C) GST-PABD beads were incubated with liposomes containing 5% PE-NBD, 85% of DOPC, and 10% PA(18:1/18:1) with the indicated % of PS (at the expense of DOPC). Liposomes with PS were compared to liposomes containing just DOPC for each PABD. Data are presented as means $\pm$ SD (of triplicate measurements from a representative experiment, n=3). Significant differences are indicated (n=3 independent experiments, * $p > 0.05$ and ** $p > 0.01$). (D) GST-PABD beads were incubated with liposomes containing 5% PE-NBD, 85% of DOPC, and 10% of PA in the presence (dark columns) or the absence (open columns) of 20 μ M calcium. Data are presented as means $\pm$ SD (of triplicate measurements from a representative experiment, n=3). For each PABD, significant differences in binding in the presence and absence of calcium are indicated (n=3 independent experiments, ** $p > 0.01$ and *** $p > 0.001$).

Figure 5: Subcellular distribution of GFP-Spo20p-PABD, GFP-Opi1p-PABD, and GFP-PDE4A1-PABD in RAW 264.7 macrophages. (A) Confocal images of macrophages expressing different GFP-PABDs and stained with the plasma membrane marker IB4, the ER marker calnexin and the Golgi marker giantin, respectively. The images shown are representative of at least 100 cells obtained with at least 5 different cell preparations. Bar = 10 μ m. Mask images highlight colocalization with the respective markers. (B) Macrophages expressing mutated GFP-PABDs. Similar observations were obtained with at least 3 different cell preparations. Bar = 10 μ m.

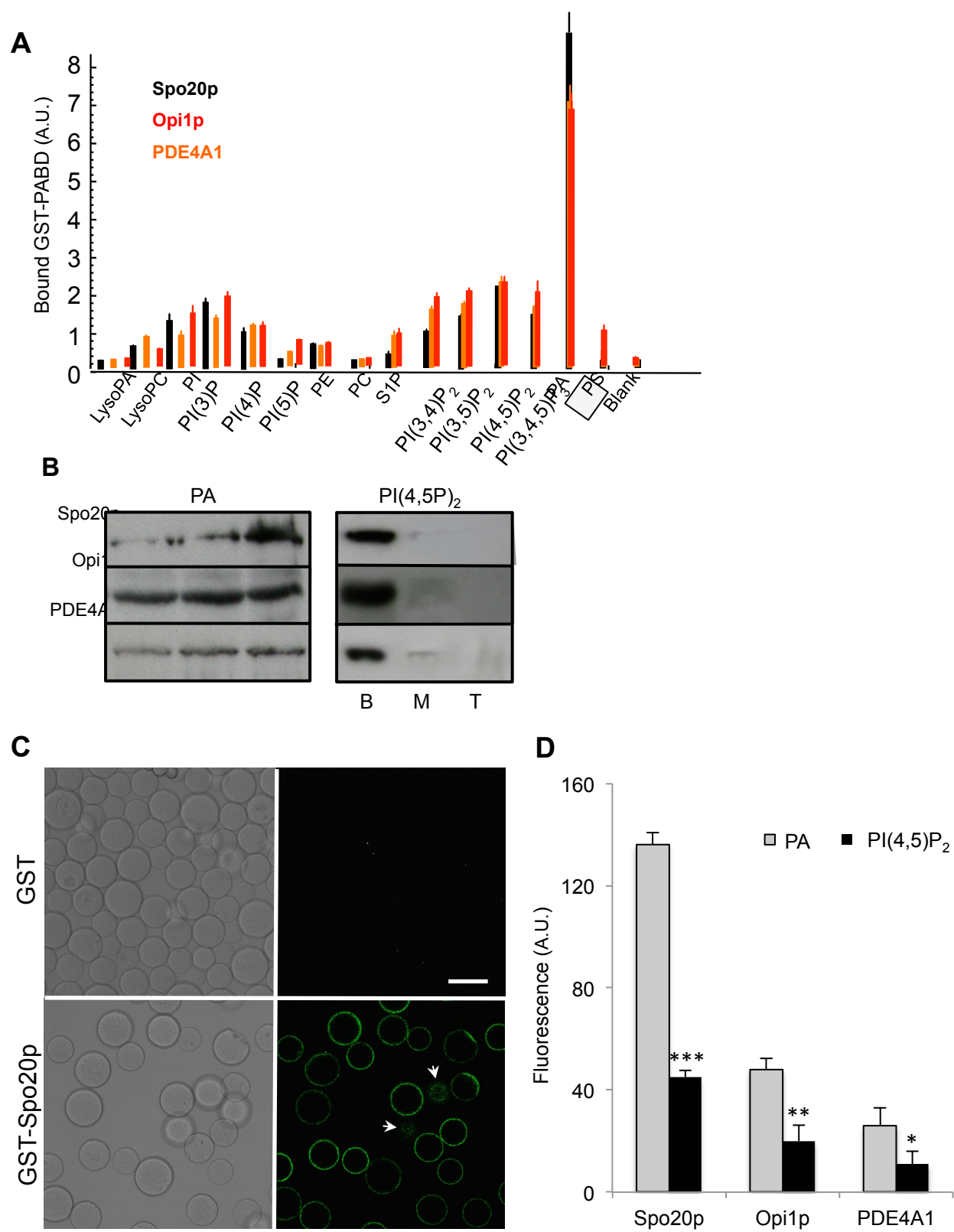
Figure 6: Subcellular distribution of GFP Spo20p-PABD, GFP-Opi1p-PABD, and GFP-PDE4A1-PABD during frustrated phagocytosis in RAW 264.7 macrophages. (A) Scanning electron microscopy images of macrophages spreading after inducing frustrated phagocytosis by shifting from 4°C to 37°C for 30 min. (B) Confocal images of macrophages expressing the different GFP-PABDs, fixed after 30 min of frustrated phagocytosis, and stained for subcellular markers as described in Fig.5A. Note that GFP-Spo20p-PABD and GFP-Opi1p-PABD (arrows) have been recruited to the plasma membrane, but GFP-PDE4A1-PABD localization is unchanged and remains concentrated in the Golgi apparatus. (C) Differential interference contrast (DIC) image and corresponding confocal images (median and basal sections) of macrophage expressing GFP-Spo20p-PABD and GFP-Opi1p-PABD after 30 min of frustrated phagocytosis. GFP-Spo20p-PABD labeled podosome-like structures at the base of the cell (arrow). The images shown are representative of at least 100 cells obtained with 3 to 5 different cell preparations. Bar = 10 μ m.

Figure 7: PA levels in subcellular membrane fractions from RAW 264.7 macrophages undergoing frustrated phagocytosis. (A) Schematic representation of the different steps from subcellular fractionation to lipidomic analysis. (B) Verification of subcellular fractionation by immunoblotting 30 μ g protein of different fractions (cytosol (Cyt), nucleus

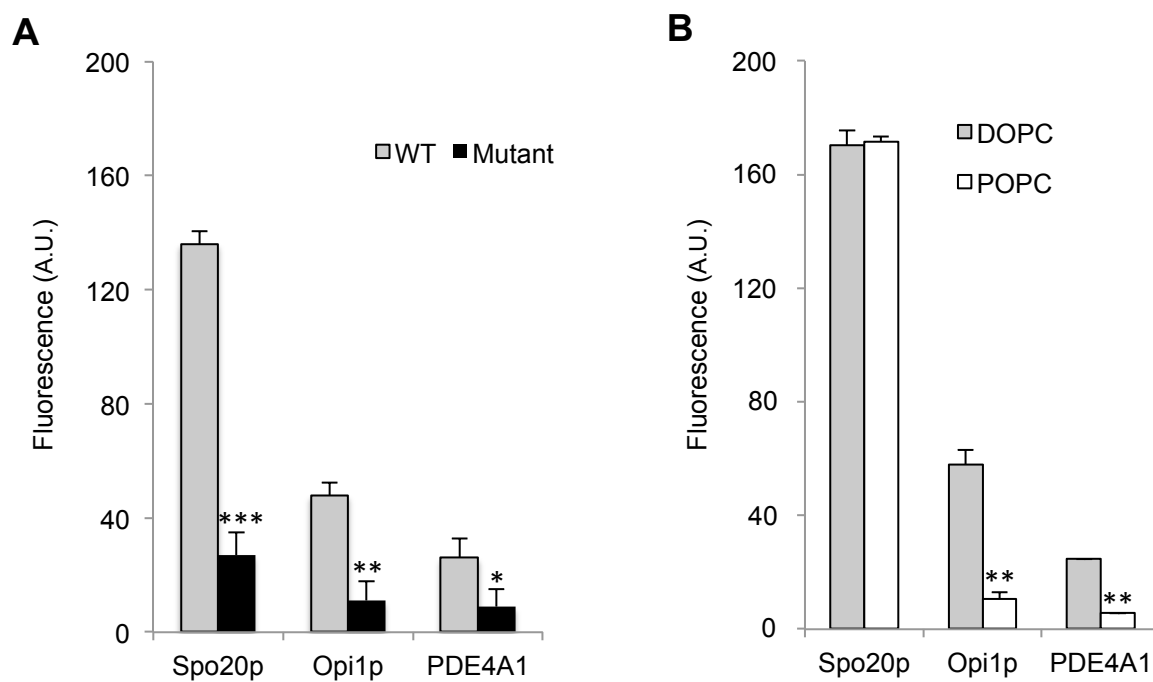
(N), plasma membrane (PM), endoplasmic reticulum (ER), mitochondria (Mito) and crude extract (Total). The following markers were used: the nuclear marker Histone H3, ER marker calnexin, cytosol marker GAPDH, mitochondrial marker HSP-60, and the plasma membrane marker Na^+/K^+ -ATPase. (C) PA levels were measured by duplicate UPLC/MS/MS analysis of two samples (each containing 200 μg of protein) from each fraction either from cells kept at 4°C (non-stimulated) or shifted to 37°C for 30 min (stimulated). Results are presented as the means $\pm$ SEM (n=3) obtained from independent experiments. Significant differences between non-stimulated and stimulated conditions for each fraction are indicated (* $p>0.05$, ** $p>0.01$, and *** $p>0.001$).

Figure 8: Binding of Spo20p-PABD and Opi1p-PABD to plasma and ER membranes obtained by subcellular fractionation (A) GST or GST-PABDs beads were incubated with plasma (PM) or ER membranes (containing ~400 μg of unsaturated lipids) for 30 min and the amount of lipid bound to the beads was estimated using a colorimetric quantification kit for unsaturated lipids. (B, C) Similar experiments were performed with plasma (PM) and ER membranes previously incubated at 37°C for 2 hrs with trypsin or proteinase K, respectively. Proteases were heat-inactivated for 1 hr at 75°C before incubating samples with GST-PABD bound to GSH beads. Results are presented as the means $\pm$ SEM (n=3) obtained from independent experiments. Significant differences for lipid binding between GST-Spo20p-PABD and GST-Opi1p-PABD are indicated (** $p>0.01$, and *** $p>0.001$). In each condition background signal measured from GST and GST-PABDs beads alone was subtracted. Inset reveals the absence of the plasma and ER membrane markers (CD64 and calnexin, respectively) as revealed by immunoblotting plasma and ER membranes after trypsin (Try) or proteinase K (PK) treatment as compared to control membranes (50 μg) incubated without protease (Ctrl).

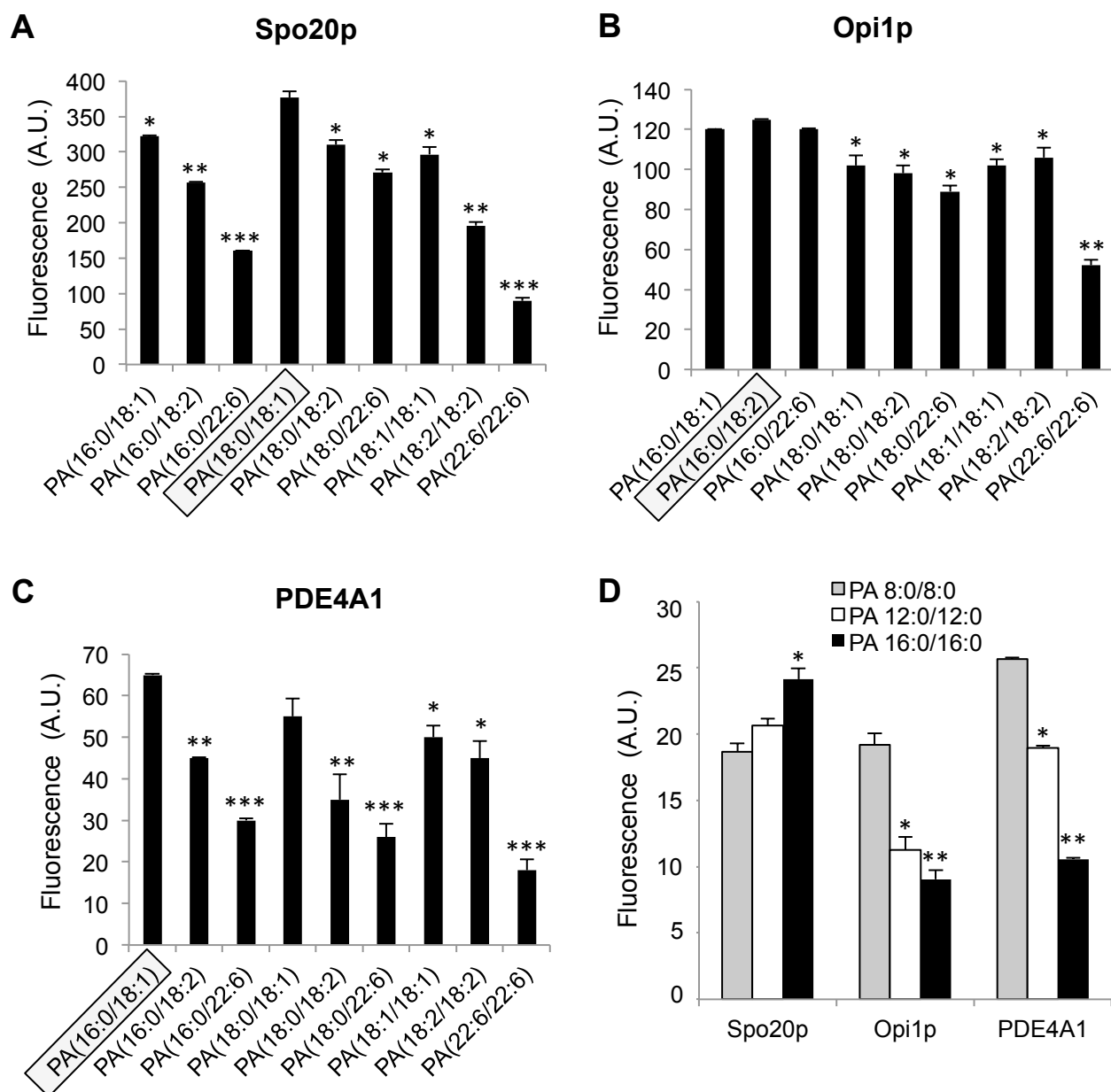
Figure 9: PA binding profile of Spo20p-PABD, Opi1p-PABD, and PDE4A1-PABD and subcellular localization in macrophages. (A) Amphipatic α helix projections of the core 18-20 amino acids of the PABD of Spo20p, Opi1p and PDE4A1 obtained with Heliquist software (<http://heliquist.ipmc.cnrs.fr>). Arrows indicate hydrophobic moments. (B) Table summarizing the data from Figures 2-4. The arrows reveal absence, positive or negative effects of the indicated liposome parameter on binding of the GST-PABDs to PA-containing liposomes. (C) Comparison of the subcellular distribution of the GFP-PABD of Spo20p, Opi1p and PDE4A1 in non-stimulated RAW 264.7 macrophages and during frustrated phagocytosis. The preferential subcellular compartment of each probe is noted first.



Kassas et al., Figure 1

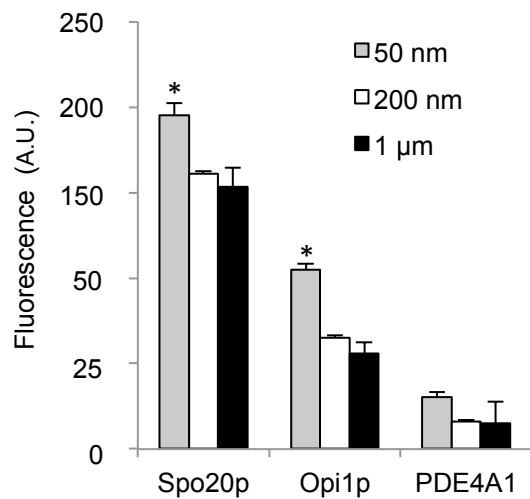


Kassas et al., Figure 2

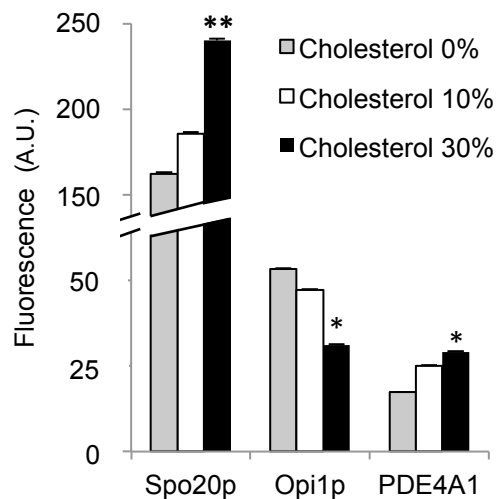


Kassas et al., Figure 3

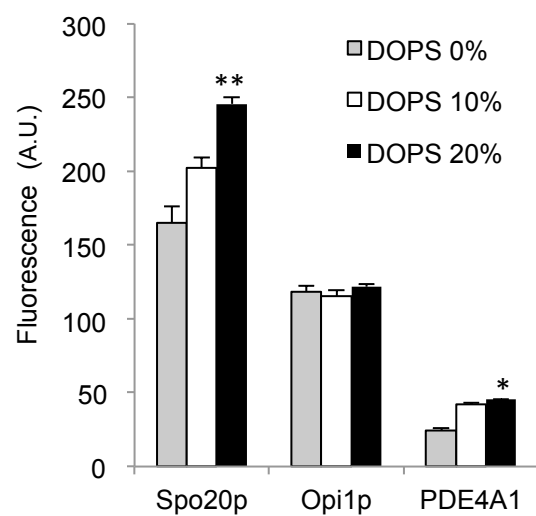
A Membrane curvature



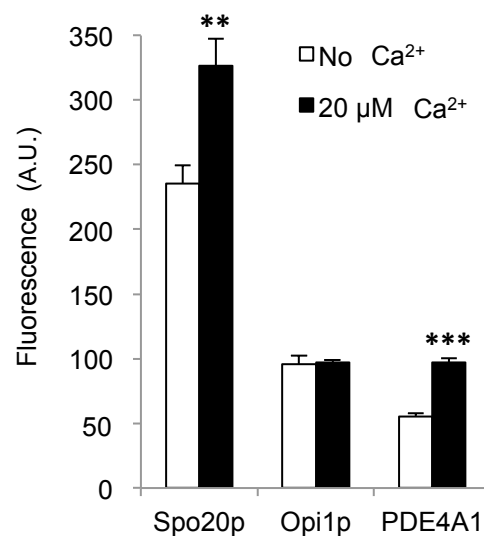
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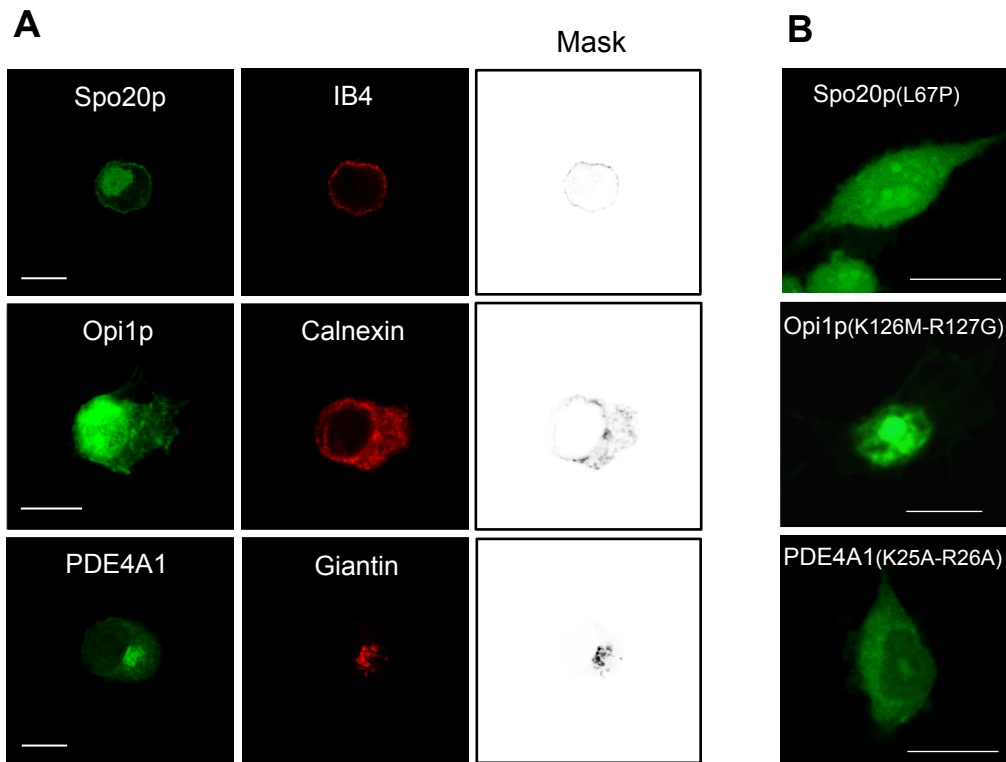
C Net charge



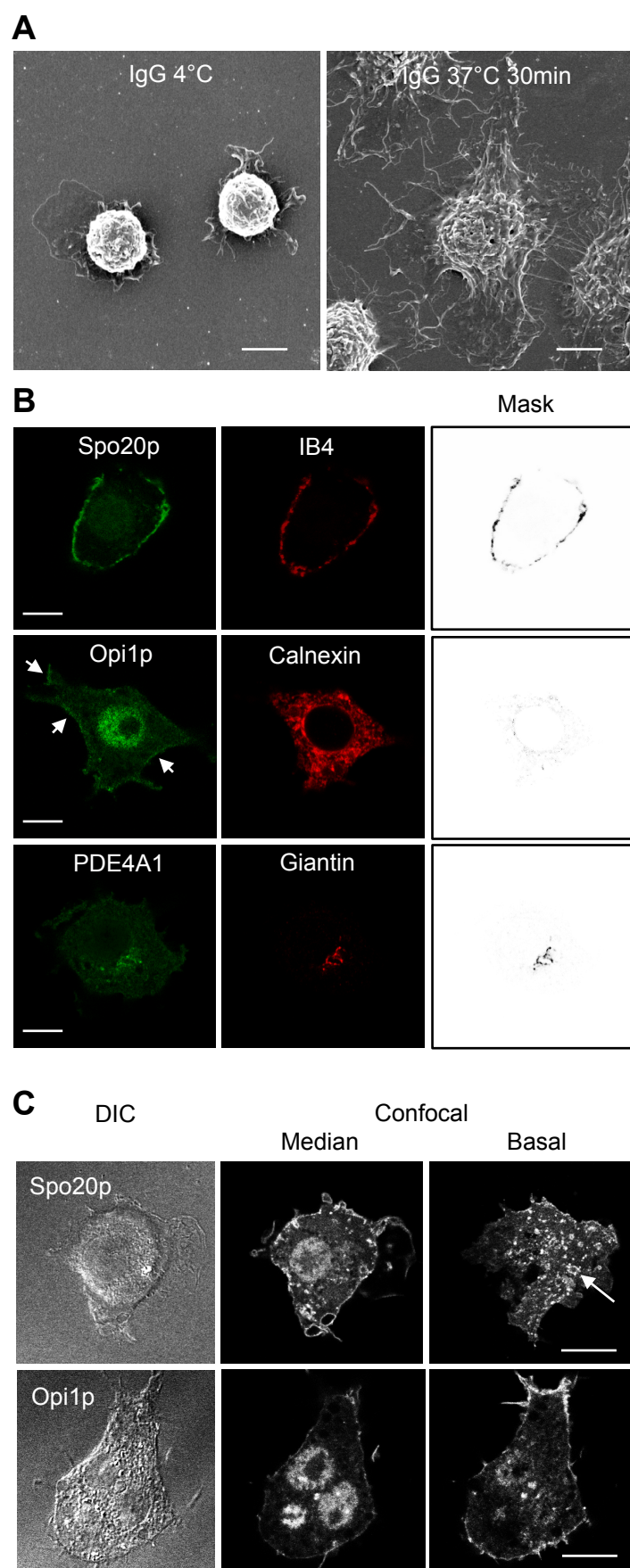
D Calcium



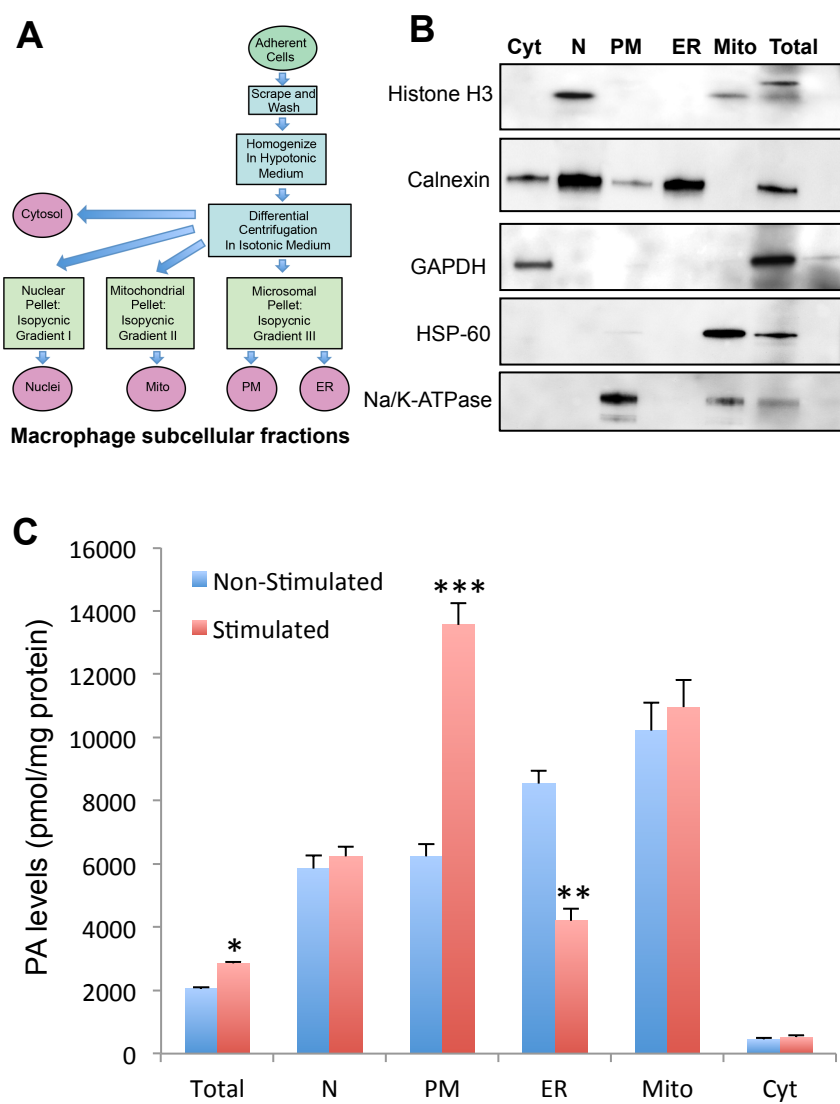
Kassas et al., Figure 4



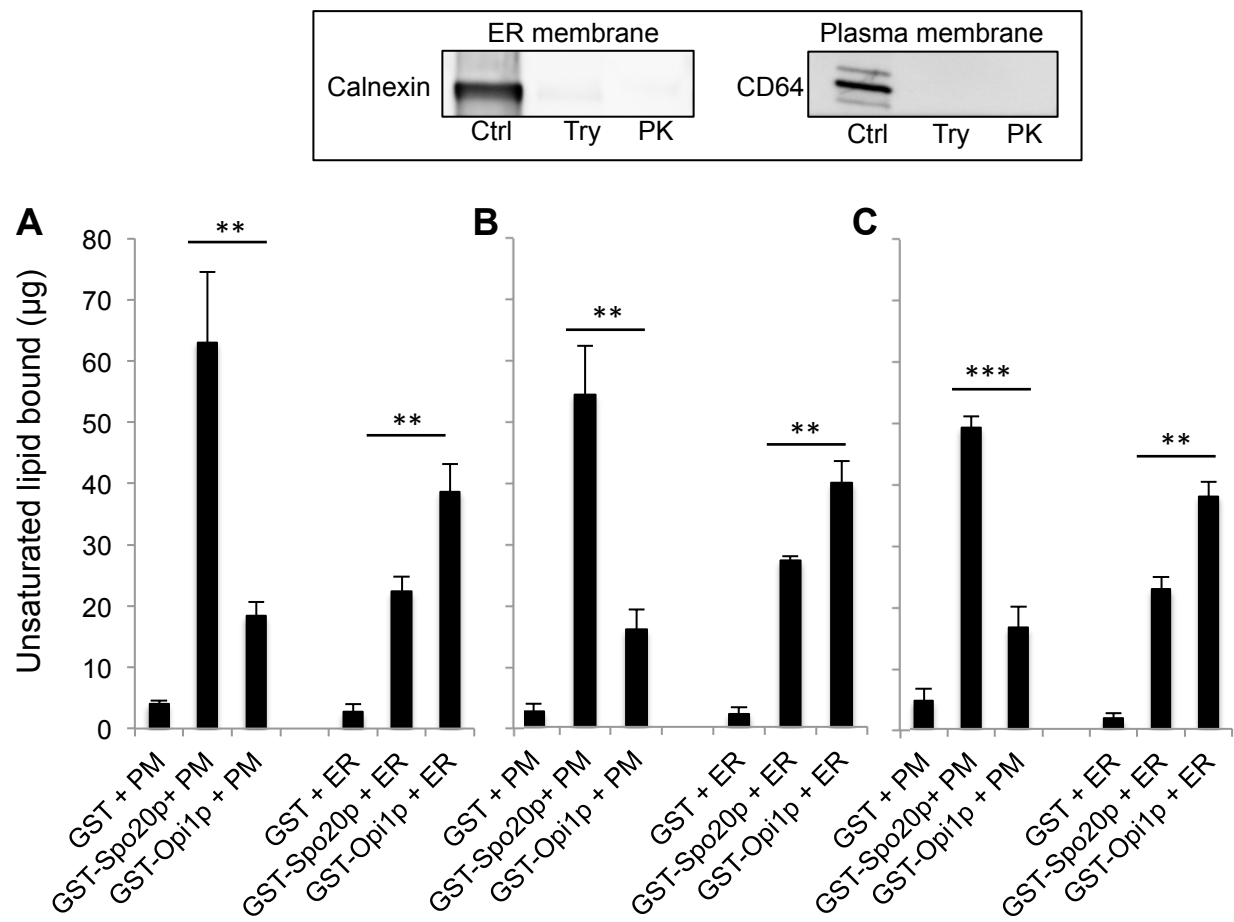
Kassas et al., Figure 5

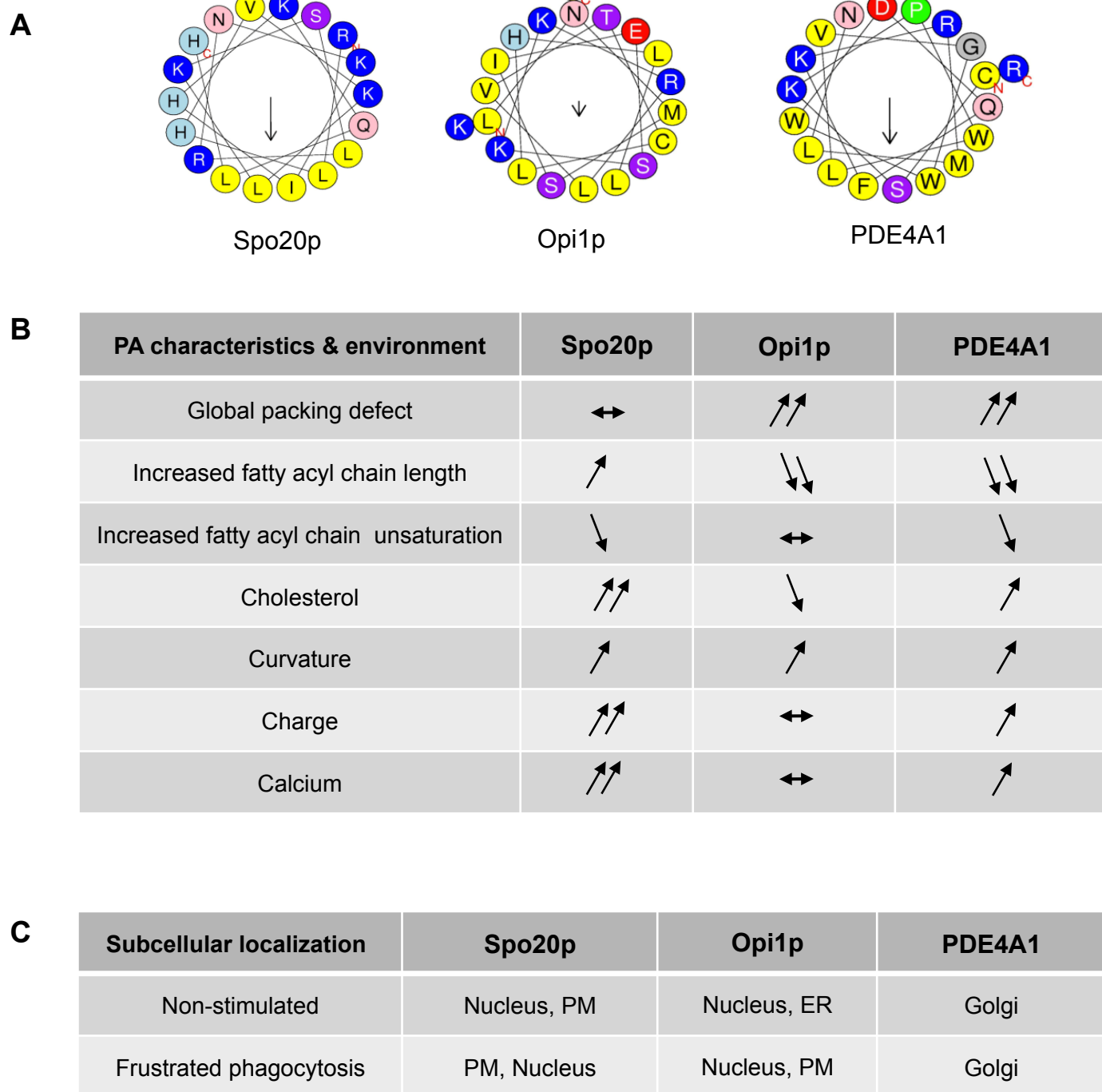


Kassas et al., Figure 6



Kassas et al., Figure 7





Comparative characterization of phosphatidic acid sensors and their localization during frustrated phagocytosis

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