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Phosphatidylinositol-4,5-Bisphosphate-Rich Plasma Membrane Patches Organize Active Zones of Endocytosis and Ruffling in Cultured Adipocytes

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A major regulator of endocytosis and cortical F-actin is thought to be phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] present in plasma membranes. Here we report that in 3T3-L1 adipocytes, clathrin-coated membrane retrieval and dense concentrations of polymerized actin occur in restricted zones of high endocytic activity. Ultrafast-acquisition and superresolution deconvolution microscopy of cultured adipocytes expressing an enhanced green fluorescent protein- or enhanced cyan fluorescent protein (ECFP)-tagged phospholipase Cδ1 (PLCδ1) pleckstrin homology (PH) domain reveals that these zones spatially coincide with large-scale PtdIns(4,5)P_2-rich plasma membrane patches (PRMPs). PRMPs exhibit lateral dimensions exceeding several micrometers, are relatively stationary, and display extensive local membrane folding that concentrates PtdIns(4,5)P_2 in three-dimensional space. In addition, a higher concentration of PtdIns(4,5)P_2 in the membranes of PRMPs than in other regions of the plasma membrane can be detected by quantitative fluorescence microscopy. Vesicular structures containing both clathrin heavy chains and PtdIns(4,5)P_2 are revealed immediately beneath PRMPs, as is dense F actin. Blockade of PtdIns(4,5)P_2 function in PRMPs by high expression of the ECFP-tagged PLCδ1 PH domain inhibits transferrin endocytosis and reduces the abundance of cortical F-actin. Membrane ruffles induced by the expression of unconventional myosin 1c were also found to localize at PRMPs. These results are consistent with the hypothesis that PRMPs organize active PtdIns(4,5)P_2 signaling zones in the adipocyte plasma membrane that in turn control regulators of endocytosis, actin dynamics, and membrane ruffling.

Phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] constitutes only about 1% of the total phospholipid in the plasma membrane but plays major roles in regulating multiple cellular processes. Classically, PtdIns(4,5)P_2 was established as the precursor of two intracellular second messengers, inositol-1,4,5-trisphosphate [Ins(3,4,5)P_3] and diacylglycerol, generated through the regulation of phospholipase C (PLC) (57). The subsequent discovery of phosphoinositide 3-kinases (9) revealed that PtdIns(4,5)P_2 was also the substrate for the synthesis of polyphosphoinositides [PtdIns(3,4,5)P_3] present in plasma membranes. Here we report that in 3T3-L1 adipocytes, clathrin-coated membrane retrieval and dense concentrations of polymerized actin occur in restricted zones of high endocytic activity. Ultrafast-acquisition and superresolution deconvolution microscopy of cultured adipocytes expressing an enhanced green fluorescent protein- or enhanced cyan fluorescent protein (ECFP)-tagged phospholipase Cδ1 (PLCδ1) pleckstrin homology (PH) domain reveals that these zones spatially coincide with large-scale PtdIns(4,5)P_2-rich plasma membrane patches (PRMPs). PRMPs exhibit lateral dimensions exceeding several micrometers, are relatively stationary, and display extensive local membrane folding that concentrates PtdIns(4,5)P_2 in three-dimensional space. In addition, a higher concentration of PtdIns(4,5)P_2 in the membranes of PRMPs than in other regions of the plasma membrane can be detected by quantitative fluorescence microscopy. Vesicular structures containing both clathrin heavy chains and PtdIns(4,5)P_2 are revealed immediately beneath PRMPs, as is dense F actin. Blockade of PtdIns(4,5)P_2 function in PRMPs by high expression of the ECFP-tagged PLCδ1 PH domain inhibits transferrin endocytosis and reduces the abundance of cortical F-actin. Membrane ruffles induced by the expression of unconventional myosin 1c were also found to localize at PRMPs. These results are consistent with the hypothesis that PRMPs organize active PtdIns(4,5)P_2 signaling zones in the adipocyte plasma membrane that in turn control regulators of endocytosis, actin dynamics, and membrane ruffling.
active, and a hypothetical signaling mechanism has emerged to account for this activity. According to this concept, localized PtdIns(4,5)P2 concentrations result from the recruitment of phosphatidylinositol 4-phosphate 5-kinase α to specialized regions of the plasma membrane in response to activation of the small G proteins Rac-1 and ARF6 (6, 16, 26, 27). However, this view was recently challenged based on biophysical characterizations of PtdIns(4,5)P2 in cell membranes (66). These new data suggest that apparent localized increases in plasma membrane PtdIns(4,5)P2 concentrations simply reflect increased amounts of total membrane constituents in these regions. Such membrane-rich domains are apparently caused by local membrane folds that are not resolved by fluorescence microscopy (13, 46, 66). Furthermore, measurements of fluorescence recovery after photobleaching suggest that lipid diffusion in the lipid bilayer is too fast to maintain these apparently PtdIns(4,5)P2-rich membrane regions (13, 42, 66). Taken together, the available data provide a confusing representation of the organization and dynamics of PtdIns(4,5)P2 in the plasma membrane and the exact role of PtdIns(4,5)P2 in regulating cellular processes.

The present studies were designed to clarify the disposition and dynamics of the endocytosis regulator PtdIns(4,5)P2 in insulin-sensitive adipocytes in three-dimensional (3D) space by using ultrafast-acquisition and superresolution deconvolution microscopy (11, 51). We focused these studies on visualizing a green fluorescent protein (GFP)-tagged probe for PtdIns(4,5)P2 in cultured 3T3-L1 adipocytes, which display active membrane trafficking, a large surface area, and a significant vertical dimension. The novel approach developed here revealed surprisingly large-scale PtdIns(4,5)P2-rich plasma membrane patches (PRMPs) with lateral dimensions exceeding several micrometers and sometimes extending from the bottom to the top of a cell. Control experiments with membrane-staining reagents revealed that local membrane folds were responsible for these PtdIns(4,5)P2-rich structures but also revealed a higher lateral PtdIns(4,5)P2 concentration in the lipid bilayer. PRMPs associate exclusively with regions of dense F actin and colocalize with highly active zones of massive endocytosis heterogeneously displayed on the cell surface. Endocytosis of transferrin and cortical F-actin formation indeed require functional PtdIns(4,5)P2. Remarkably, PRMPs also appear to define active regions of membrane ruffling induced by the expression of unconventional myosin 1c (Myo1c). Taken together, the data define a novel plasma membrane domain in cultured adipocytes that concentrates PtdIns(4,5)P2 in 3D space to apparently regulate important plasma membrane functions.

**MATERIALS AND METHODS**

**DNA constructs.** Previously characterized plasmid constructs (pEYFP-N1; Clontech, Palo Alto, Calif.) containing the pleckstrin homology (PH) domain of PLCδ1 linked to enhanced GFP (EGFP) (EGFP/PLCδ1-PH) or a PtdIns(4,5)P2-binding-deficient mutant (R40L) were generous gifts from Tamás Balla (National Institutes of Health, Bethesda, Md.). The insert of the PLCδ1 PH domain and the corresponding mutant were amplified by PCR with primers 5’T-TGACCTCGCAATTACGGCATGGACT-3 (5’T-ATGACCTTGCTTATGCTGTCAGC-3). Following digestion with EcoRI and XbaI, the purified PCR products were ligated to the same multiple cloning sites in plasmid pEYFP-C1 (Clontech). The correct inserts were verified by sequencing. Plasmid pEYFP-C1 (Clontech) containing Myo1c was previously characterized (4). The pcDNA3.1 construct containing the human transferrin receptor (hTfRc) was a generous gift from Haley Melikian (University of Massachusetts Medical School).

**Cell culture and fluorescent labeling of the plasma membrane.** 3T3-L1 fibroblasts (American Type Culture Collection, Manassas, Va.) were grown to confluence and differentiated as previously described (47). Day 6 to day 8 differentiated adipocytes were electroporated (47) with 50 μg of EGFP/PLCδ1-PH, ECFP/PLCδ1-PH (enhanced cyan fluorescent protein [ECFP] linked to the PH domain of PLCδ1) or EYFP/Myc1 (enhanced yellow fluorescent protein [EYFP] linked to Myo1c) plasmid DNA and subsequently plated on no. 1.5 coverslips (Thomas Scientific, Swedesboro, N.J.). Transfected cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) for 12 h before being switched to DMEM without fetal calf serum (serum-starved medium). Penicillin at 50 U/ml and streptomycin at 50 μg/ml were always included to prevent bacterial contamination.

After >2 h of serum starvation, live-cell microscopy was carried out at 37°C with KRH buffer (125 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.2 mM MgSO4, 2 mM sodium pyruvate, 25 mM HEPES [pH 7.4], 0.2% [wt/vol] bovine serum albumin). Insulin stimulation of serum-starved cells was carried out at 37°C with 100 nM human insulin (Eli Lilly, Indianapolis, Ind.). Alternatively, cells were fixed in 4% (wt/vol) formaldehyde at room temperature for 12 min and preserved in ProLong immersion medium for superresolution deconvolution microscopic studies. Following permeabilization of fixed cells with 0.5% (vol/vol) Triton X-100, F-actin was stained with rhodamine (RhD)-conjugated phalloidin (RhD-PhD). Alternatively, endogenous clathrin heavy chains were targeted with a rabbit polyclonal antibody developed in the laboratory of Silvia Corvera (University of Massachusetts Medical School), followed by immunofluorescence staining with an RhD-conjugated goat anti-rabbit secondary antibody. Depending on the experiment, plasma membrane staining with ~5 μg of Alexa488- (Alx488)- or Alexa594 (Alx594)-conjugated concanavalin A (ConA)/ml for 2 to 3 min, ~1 μM BODIPY-HPC for ~30 min, or ~1 μM DiIC18 (~5 min was carried out with live or fixed adipocytes and at 4°C (to inhibit endocytosis), room temperature (for fixed cells), or 37°C (for live cells). All fluorescent reagents and ProLong immersion medium were purchased from Molecular Probes (Eugene, Oreg.).

**Transferrin endocytosis.** Day 4 differentiated 3T3-L1 adipocytes were coelectroporated with 50 and 150 μg of plasmid DNAs containing hTfRc and EGFP/PLCδ1-PH sequences, respectively. The cells were cultured overnight at 37°C. The adipocytes were washed once with KRH buffer, cooled to 4°C, and incubated at 4°C for 1 h in KRH buffer with 20 μg of Alx488-conjugated transferrin (Alx488-TF; Molecular Probes)/ml. Subsequently, the cells were warmed to 37°C and incubated for 15 min to allow Alx488-TF uptake. Another set of adipocytes was electroporated with 50 μg of hTfRc DNA alone and treated with 60 μM latrunculin B (LB) for 2 h at 37°C before Alx488-TF labeling and uptake experiments were carried out. The cells were washed three times with ice-cold phosphate-buffered saline and immediately fixed in 4% formaldehyde. Fixed cells were labeled with RhD-PhD for F-actin.

**Microscopy.** The laser-illuminated ultrafast-acquisition microscope and its applications to investigations of cellular dynamics have been described in detail elsewhere (47, 51). Coupled with a Nikon 60×1.40-numerical-aperture (NA) oil objective, the setup was able to acquire image stacks with a low pixel resolution of 333 nm and a spacing of 250 nm. This spatial combination produced ripple-like patterns in the deconvolved images (see Fig. 5, 7 to 9, and 12) that should be regarded as continuous regions. High-magnification imaging (see Fig. 5, 7 to 9, and 12) with a pixel size of 66 nm and a spacing of 250 nm was carried out by using a Zeiss Axiosvert 200 M microscope equipped with a 100×1.40-NA oil objective. Image stacks were deconvolved with the superresolution deconvolution algorithm developed by Carrington et al. (11). On the high-magnification images, the above-mentioned ripple-like patterns are almost not noticeable.

Image rendering and analysis were carried out using Data Analysis and Visualization Environment (40) and other software tools developed by the Biomedical Imaging Group at the University of Massachusetts Medical School (http://invitro.umassmed.edu). Typically, the dynamic range (i.e., the minimum to maximum pixel intensity ranges) of 12- and 14-bit images obtained from the ultrafast-acquisition and Zeiss microscopes, respectively, was first linearly mapped to the full scale (i.e., 0 to 255) of an 8-bit gray or false-color intensity scale. The images were further rescaled to a narrower intensity range (i.e., contrast enhanced). The deformable snake algorithm (71) was modified slightly for use with our data and was used to automatically find cell boundaries (see Fig. 3 and 4). With the aid of the Align program (http://invitro.umassmed.edu), images of the same cell obtained with different fluorescence channels were visualized and aligned to within ~3 pixels of each other.

Filter sets were purchased from Chroma Technology Co. (Burlington, Vt.) for imaging ECFP (i.e., CFP channel; filter set 31044 v2), EGFP-Alx488 (i.e., GFP-
channel; 41001), Alx488-ConA/Alx488-TF (i.e., YFP channel; CZ2014) fluorescence. Cross talk among fluorescence channels was characterized with specimens from single-fluorophore labeling and was found to be negligible. Occasionally, Alx488-ConA/Alx488-TF fluorescence was deliberately imaged in the YFP channel in three-color colocalization studies (see Fig. 5, 9, and 10) to avoid fluorescence bleedthrough from the CFP channel.

Quantitative image analysis. A fluorescence image (I) can be approximated as the convolved product of the fluorescent specimen (S(r)), the point spread function (PSF), and the spatial detection efficiency function (D(r)) of the microscope, so that I(r) = D(r) * S(r) * PSF(r). For the green (F_G) and red (F_R) fluorescence images of the same specimen, ΔF = F_G - F_R = [D(r)G × S(r)G × PSF_G × dr × dr'] - [D(r)R × S(r)R × PSF_R × dr × dr']. In this study, the green image is associated with the EGFP/PLCδ1-PH label, and the red one is associated with the BODIPY-HPC/Alx594-ConA probe (see Fig. 3 and 4). D(r) is a property of the microscope system, and when F_G and F_R are appropriately normalized (see Results), ΔF(r) = D(r)G - D(r)R. The measured PSFs are very similar (i.e., PSF_G ≈ PSF_R), consistent with the negligible chromatic effect observed in the deconvolved images. Consequently, ΔF ≈ |D(r)G - D(r)R| × S(r)G/PSF_G × dr × dr'. Thus, ΔF reflects only the difference in the fluorescent probes present in the specimens, as it should. This scenario was confirmed in a control experiment, in which ratiometric analysis of an EGFP/PLCδ1-PH fluorescence image and its counterpart detected in the YFP channel by use of bleedthrough signals revealed that small systematic error was introduced by the microscope system (see Fig. S1 at http://invitro.umassmed.edu/~sh/supplements/supplements.html).

RESULTS

PtdIns(4,5)P_2 is concentrated in PRMPs in live 3T3-L1 adipocytes. In this study, we used an ultrafast-acquisition deconvolution microscopic technique to image the real-time distribution of PtdIns(4,5)P_2 in live 3T3-L1 adipocytes. PtdIns(4,5)P_2 was visualized by transient expression of a fusion protein linking EGFP to the PH domain of PLCδ1 (EGFP/PLCδ1-PH) (Fig. 1) (3). With this technique, a typical 3D image consisting of 81 z sections (~20 μm thick) (Fig. 1A or C) can be acquired in about 1 s. Because of the high efficiency of utilizing fluorescence photons and thus the relatively low excitation power used, the ultrafast-acquisition deconvolution microscope is least affected by photobleaching. With wide-field illumination, it is also the fastest way to acquire 3D images. These characteristics proved to be important for capturing real-time fluorescence images that closely represent the distribution and dynamics of PtdIns(4,5)P_2 in live cells.

PtdIns(4,5)P_2 is primarily localized to the plasma membrane of cultured adipocytes (Fig. 1), consistent with previous observations obtained when EGFP/PLCδ1-PH was used as the fluorescent marker in other cell lines (6, 56, 66, 67). In contrast, the EGFP/PLCδ1-PH mutant deficient in PtdIns(4,5)P_2 binding (i.e., R40L) (67) shows no plasma membrane localization but a mostly uniform fluorescence distribution in the cytoplasm of 3T3-L1 adipocytes (see Fig. S2 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). Surprisingly, we found that PtdIns(4,5)P_2 is enriched in distinct regions at the adipocyte surface (Fig. 1; see also movie 1 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). Large-scale PtdIns(4,5)P_2-rich membrane domains (red regions in Fig. 1A, C, and D) with lateral dimensions exceeding several micrometers are observed and sometimes extend through almost the entire height of the cell (Fig. 1A). These PtdIns(4,5)P_2-rich domains are found in both serum-starved cells (Fig. 1A and D) and insulin-stimulated cells (Fig. 1C). The fact that most of these domains are organized perpendicular to the coverslip-attached plasma membrane suggests that they are not artifacts of photobleaching during image acquisition. These domains are visualized with 8-bit, contrast-enhanced, false-color intensity scales (intensity scale bar in Fig. 1A; see Materials and Methods) and are the approximation of their counterparts shown in the corresponding 8-bit gray-scale image without contrast enhancement (inset in Fig. 1A). Within these domains, gradients of PtdIns(4,5)P_2 distribution also exist, as exemplified by the 2D intensity plot (Fig. 1B; see also movie 1 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) of the 45th z section of the cell shown in Fig. 1A (yellow outline). Since the full widths at half maximum of the intensity peaks shown in Fig. 1B (and in movie 1 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) mostly exceed several micrometers, these large-scale PtdIns(4,5)P_2-rich membrane structures are clearly different from “membrane rafts,” whose sizes are believed to be under the resolution of optical microscopy (>200 nm) (30). Instead, the physical sizes of these domains are close or larger than those of membrane ruffles and phagosomes, within which PtdIns(4,5)P_2 enrichment has also been observed (6, 27). Thus, we designate these structures “membrane patches.” In live adipocytes, we found PRMPs colocalized with membrane ruffles (yellow arrows in Fig. 1D) and in apparently thickened membrane regions (red arrow in Fig. 1D).

Recent evidence has surprisingly indicated that PtdIns(4,5)P_2-rich membrane domains may be caused entirely by membrane folds not resolved by fluorescence microscopy (13, 66). These findings challenge the concept that PtdIns(4,5)P_2 is laterally segregated in the 2D lipid bilayer. To test this possibility, we labeled live adipocytes expressing EGFP/PLCδ1-PH with Alx594-ConA and carried out time-dependent, dual-color deconvolution microscopic measurements (Fig. 2). Alx594-ConA has been used as a reagent for plasma membrane staining (7), and its fluorescence intensity was used as an indicator of local membrane density in this experiment (see below). We found that PRMPs in a cultured adipocyte undergo dynamic changes with (Fig. 2D and E) and without (Fig. 2A to C) insulin stimulation and that these changes closely mirror the dynamic distribution patterns for Alx594-ConA at the corresponding time points (Fig. 2F to J; see also movie 2 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). The fact that PRMPs dynamically colocalize with membrane-rich domains stained with concentrated Alx594-ConA suggests that the apparent PtdIns(4,5)P_2 enrichments result at least partially from increases in local membrane content (see below) (66).

The time-dependent PRMP reorganization shown in Fig. 2 (and movie 2 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) is associated with cellular morphology changes (i.e., the top portion of the cell expanded during image acquisition) but is not correlated with insulin stimulation. In fact, large-scale PRMP dynamics are not induced by insulin but are associated with dramatic changes in cellular morphology (see movie 3 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). In contrast, most live adipocytes (i.e., >10 cells) examined by ultrafast-acquisition microscopy are sedimentary and show no change in cell shape. Correspondingly, PRMPs are very stable (i.e., a half-life of >0.5 h) in these cells regardless of insulin stimulation (see movie 4 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). How-
FIG. 1. Large-scale PRMPs on the surface of living 3T3-L1 adipocytes. (A, C, and D) 3D image stacks of adipocytes transiently expressing EGFP/PLC61-PH were acquired with an ultrafast-acquisition microscope (see Materials and Methods), and representative deconvolved images were projected within 3D grids measuring 42 by 43 by 20 μm. These cells are composed of 81, 81, and 30 z sections, respectively, which are stacked successively along the optical z axis. Each z section represents a 0.25-μm slice of cells. The cells are oriented in such a way that the bottom membranes attached to the coverslip are closest to the plane containing the origin (O). All cells were serum starved for >2 h, and the cell in panel C was subsequently insulin stimulated for 10 min at 37°C. To facilitate visualization of the PtdIns(4,5)P₂ spatial distribution, the dynamic ranges (i.e., minimum to maximum pixel intensity ranges) of the original 12-bit images are linearly mapped to 8-bit false-color intensity scales. The images are further contrast enhanced between their respective background noise levels and 10 times above those levels (e.g., the false-color intensity scale bar in panel A). Thus, the image in panel A was rescaled at between 10 and 100, and those in panels C and D were rescaled at between 20 and 200. The resulting color images (e.g., panel A) were the first approximations of their 8-bit gray-scale counterparts without contrast enhancement (e.g., inset in panel A). Only the bottom section (i.e., z1 to z30) of the cell in panel D is shown; yellow arrows indicate membrane ruffles, while the red arrow indicates apparently thickened membrane regions. Ripple-like patterns in the 3D images (most apparent in the red regions in panels A and C) are related to the axial-to-lateral magnification ratio used for the ultrafast-acquisition microscope and should be regarded as continuous regions (see Materials and Methods).(B) Full-scale (i.e., 0 to 255) 8-bit 2D intensity plot of the 45th z section of the cell in panel A (yellow outline in panel A). (See movie 1 at http://invitro.umassmed.edu/~sh/supplements/supplements.html for a complete set of 2D intensity plots.)
However, the ultrafast-acquisition microscopy technique used here sacrifices resolution for speed (see Materials and Methods) and may miss structural changes occurring on smaller spatial scales (35). Therefore, structural details associated with PRMPs were further examined in fixed adipocytes (see Fig. 5, 7 to 9, and 12) by using high-magnification (i.e., 66 nm/pixel) imaging combined with a superresolution deconvolution algorithm, which achieves a lateral resolution approaching ~100 nm (11). Alternatively, the frequency and size of membrane ruffles can be enhanced by overexpression of unconventional Myo1c, thus enabling real-time examination of their spatial relationship to PRMPs (see Fig. 13; see also movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html).

**Mechanisms of PtdIns(4,5)P_2 enrichment in PRMPs.** To further investigate the mechanisms that concentrate PtdIns(4,5)P_2 in PRMPs, we carried out a quantitative ratiometric analysis (see Materials and Methods) of images of doubly-labeled adipocytes (Fig. 3 and 4). Three membrane-staining reagents, DiIC18, BODIPY-HPC, and Alx594-ConA, were tested for their suitability for indicating local lipid or membrane density. The former two are lipophilic dyes that are preferentially partitioned into lipid-ordered and lipid-fluid phases, respectively (37), and the latter mostly binds to cell surface glycoproteins. Surprisingly, only 57% of live adipocytes (34 out of 60) are stained with DiIC18, and the percentage decreases to 35% for cells expressing EGFP/PLC_δ1-PH (19 out of 54). The significance of these results is not clear yet. However, since DiIC18 has been shown to stain a variety of cells (36, 66), this observation does indicate that the lipid composition of the adipocyte plasma membrane is unique in that it partially excludes DiIC18 labeling.

In contrast, it was found that both BODIPY-HPC and Alx594-ConA stain all 3T3-L1 adipocytes, whether or not the cells express EGFP/PLC_δ1-PH. Single-plane images of live adipocytes were captured at each time point (Fig. 2), and deconvolved false-color images were rescaled between 10 and 100 (Fig. 1). Scale bar, 5 μm.

**FIG. 2.** PRMPs dynamically colocalize with lipid-dense plasma membrane domains on the surface of living adipocytes. The plasma membrane of a living adipocyte transiently expressing EGFP/PLC_δ1-PH (A to E) was labeled with Alx594-ConA (F to J). Dual-color ultrafast-acquisition microscopy of this cell was carried out every 20 s for 20 min at 37°C. Image stacks consisting of 81 z sections were acquired with the EGFP and Alx594 fluorescence channels in an interlaced fashion, with the corresponding z sections in these two channels being acquired sequentially and separated by ~5 ms. Only side views of the plasma membrane are shown at representative time points (e.g., A and F at 0 min), and 100 nM insulin was added at 10 min (i.e., immediately after C and H). Deconvolved false-color images acquired with the EGFP (A to E) and Alx594 (F to J) fluorescence channels were rescaled between 10 and 100, respectively (Fig. 1). The arrow in panel F indicates part of a fibroblast in the foreground of panels F to J; this fibroblast is labeled with Alx594-ConA but does not express EGFP/PLC_δ1-PH. Scale bar, 5 μm.
cells obtained with both the EGFP (Fig. 3A) and the BODIPY-HPC (Fig. 3B) fluorescence channels show little fluorescence bleaching. With this method, only fluorescence variations within the in-focus plane (i.e., a plane with a thickness of ~1.0 μm for a 1.40-NA oil objective) are resolved, and out-of-focus fluorescence contributes to a mostly smooth background (53). Measurements were obtained for 20 cells, and a representative example is shown in Fig. 3. A deformable snake algorithm (71) was used to automatically find the cellular boundary (shown only in panel A as a red line). D) Plot of normalized fluorescence intensities (i.e., $I_{\text{EGFP}}/I_{\text{EGFP}}$ and $I_{\text{BODIPY}}/I_{\text{BODIPY}}$; see Results) along the cell boundary (green line, EGFP/PLCδ1-PH fluorescence; red line, BODIPY-HPC fluorescence). Pix. Int., pixel intensity. Blue line, baseline. (E) Ratiometric intensities (i.e., $I_{\text{EGFP}}/I_{\text{BODIPY}}$). (C) Corresponding 14-bit ratiometric image [i.e., panel C = (panel A $\cdot 10^4$)/(panel B $\cdot \text{NF}$)]. The normalization factor (NF) is defined as $I_{\text{EGFP}}/I_{\text{BODIPY}}$. The image in panel C was further rescaled at between $10^4$ and the maximum pixel intensity so that only an $I_{\text{EGFP}}/I_{\text{BODIPY}}$ ratio $>1$ is visible. Peak EGFP fluorescence intensities corresponding to PtdIns(4,5)P$_2$-rich membrane domains are labeled 1 to 9 in panels A and D. However, the cross-hatched region in panel E could also represent a broad peak. The arrow in panel A indicates the starting point of the line intensity profile (clockwise). BODIPY-HPC staining was carried out at 0°C, and images were acquired at 8 to 10°C to inhibit constitutive endocytosis. Scale bar, 5 μm.

FIG. 3. Quantitative image analysis of a live adipocyte expressing EGFP/PLCδ1-PH (A) and labeled with the membrane-staining dye BODIPY-HPC (B). A deformable snake algorithm (71) was used to automatically find the cellular boundary (shown only in panel A as a red line).
The EGFP and BODIPY-HPC fluorescence intensities ($I_{\text{EGFP}}$ and $I_{\text{BODIPY}}$, respectively) are normalized to their respective global minimums (IGMs) and are plotted in Fig. 3D. Since membrane regions associated with IGMs are the least likely places for both membrane folding and lateral PtdIns(4,5)P$_2$ segregation, the normalized BODIPY signal (i.e., $I_{\text{BODIPY}} / I_{\text{BODIPY}}$) reflects the relative degree of local membrane density elsewhere, while the normalized EGFP signal (i.e., $I'_{\text{EGFP}} = I_{\text{EGFP}} / I_{\text{EGFP}}$) accounts for both local membrane density and additional lateral PtdIns(4,5)P$_2$ accumulation in the 2D lipid bilayer. Consequently, $\Delta' = [(I'_{\text{EGFP}} - I'_{\text{BODIPY}})] \times 100$ is a good approximation of local PtdIns(4,5)P$_2$ enrichment beyond what can be accounted for by an increase in local membrane content (see Fig. 4).

**FIG. 4.** Quantitative image analysis of a fixed adipocyte expressing EGFP/PLC51-PH (A) and labeled with the membrane-staining probe Alx594-ConA (B). (F) Plot of normalized fluorescence intensities (green line, EGFP fluorescence; red line, Alx594 signal) along the cell boundary (i.e., red lines in panels A and B). Pix. Int., pixel intensity. Blue line, baseline. (C and G) Ratiometric intensities (G) and corresponding ratiometric image (C) generated as described in the legend to Fig. 3. (D and E) Magnified views of the boxed regions (box shown only in panel B) in panels A and B, respectively. Peak EGFP fluorescence intensities corresponding to PtdIns(4,5)P$_2$-rich membrane domains are labeled 1 to 9 in panels A and F. Green and red arrows in panel A indicate the starting and ending points of the line intensity profile (clockwise), respectively. Consequently, a portion of the cell boundary suspected of being broken is excluded from data analysis. Scale bar, 5 μm.
Materials and Methods). In addition, the normalization procedure also accounts for different levels of expression of EGFP/PLC61-PH in individual cells.

From Fig. 3D, it is clear that PtdIns(4,5)P2-rich (i.e., EGFP/PLC61-PH labeling; green line) and lipid-dense (i.e., BODIPY-HPC labeling; red line) membrane domains (labeled 1 to 8 in Fig. 3A and D) exclusively colocalize, a finding which is also apparent from a comparison of the fluorescence images in Fig. 3A and B. This colocalization is found in all 20 cells examined and reaffirms the conclusion derived from Fig. 2 that PtdIns(4,5)P2 is enriched in the 3D space of PRMPs through a mechanism involving local membrane folding. Furthermore, except for domain 1, peak EGFP fluorescence intensities are all higher than their BODIPY-HPC counterparts. An average Δ′ of 45% ± 10% (mean and standard error of the mean) was obtained for all eight peaks, indicating additional PtdIns(4,5)P2 enrichment in the PRMPs beyond the increase in local membrane density. However, it was also observed that IEGFP is globally higher than IBODIPY, except in the vicinity surrounding peaks 1 and 8. In fact, the average EGFP intensity is 23% ± 1% higher than that of its BODIPY-HPC counterpart. These results could suggest that a nonlinear baseline instead of the linear baseline depicted in Fig. 3D (blue line) should be used to estimate relative peak heights. Thus, a peak height should be defined by the baseline established by its associated valleys. To test this hypothesis, a ratiometric intensity profile \((I_{\text{EGFP}}/I_{\text{BODIPY}})\), which is independent of the methods of fluorescence normalization (see above), is plotted in Fig. 3E. It clearly shows, for peaks 2, 3, 5, and 7, higher magnitudes of EGFP fluorescence above the noisy baselines surrounding these peaks (Fig. 3E). This effect is best visualized in the ratiometric image (Fig. 3C), in which the higher EGFP/PLC61-PH intensities are clearly visible above the background noise. Finally, the exact value of Δ′ is dependent on how a fluorescence peak is defined. For example, the cross-hatched region in Fig. 3E can be regarded as a broad peak. In this case, the calculated Δ′ will be between 23% ± 1% and 45% ± 10%.

The above-mentioned considerations demonstrate that the average Δ′ of 45% ± 10% should be regarded as the upper limit of lateral PtdIns(4,5)P2 enrichment in PRMPs. Similarly, an average upper limit of 46% ± 13% was calculated from a total of 55 PRMPs examined for four representative cells. However, quantitative analysis (Fig. 3) assumes that there is no interaction between the BODIPY-HPC and EGFP/PLC61-PH labels, because the data (Fig. 3) can also be explained by a nonlinear exclusion mechanism, in which a higher local level of EGFP/PLC61-PH progressively inhibits BODIPY-HPC labeling. Therefore, we tested another fluorescent probe that binds to the plasma membrane via a different mechanism (see below).

ConA binds to oligosaccharide residues on the outer leaflet of plasma membranes, while EGFP/PLC61-PH targets PtdIns(4,5)P2 on the inner leaflet. Therefore, these two membrane probes should not interfere with each other. Furthermore, unlike lipophilic dyes, ConA can be effectively cross-linked in formaldehyde-fixed samples. Thus, fluorescently labeled ConA is the reagent of choice for subsequent super-resolution deconvolution microscopic studies of fixed adipocytes (Fig. 5). However, ConA can only indirectly probe local lipid density through its interaction with membrane-bound glycoproteins. Thus, we found various degrees of spatial divergence between the ConA- and EGFP/PLC61-PH-rich membrane domains in fixed adipocytes. Nevertheless, where these two probes completely colocalize in the plasma membrane (Fig. 4), we found that the normalized Alx594-ConA and EGFP/PLC61-PH intensity profiles are well aligned (Fig. 4F).

That is, differences in fluorescence intensities occur only in the peak positions (labeled 1 to 9 in Fig. 4A and F), and the valleys associated with these peaks overlap (Fig. 4F). Therefore, additional PtdIns(4,5)P2 enrichment occurs only in PRMPs. The average Δ′ was calculated to be 48% ± 8% (excluding peak 4); this value agrees very well with the average Δ′ of 46% ± 13% estimated with BODIPY-HPC as the membrane stain (Fig. 3). These results, together with the ratiometric intensity profile (Fig. 4G) and the ratiometric image (Fig. 4C), indicate that there is additional PtdIns(4,5)P2 enrichment in PRMPs above that accounted for by an increase in local membrane content. Taken together, the results derived from Fig. 3 and 4 support a mechanism in which PtdIns(4,5)P2 is somehow concentrated in the lipid bilayer of PRMPs in addition to its increased concentration in 3D space due to local membrane folding.

We noted that peak 4 in Fig. 4 is unique in that it has a high EGFP/PLC61-PH fluorescence intensity but no corresponding spike in Alx594-ConA labeling. This is because the deformable snake algorithm localizes the maximum local EGFP fluorescence (Fig. 4D) to just beneath the plasma membrane defined by extracellular Alx594-ConA labeling (Fig. 4E). Thus, fluorescence signals associated with these two reporters do not colocalize exactly in this region. The PtdIns(4,5)P2-containing structures immediately beneath the plasma membrane were examined in detail in Fig. 7 to 9.

The data reported above indicate that PRMPs are PtdIns(4,5)P2- and membrane-rich structures on the adipocyte cell surface. The estimated upper limit of 46% ± 13% for PtdIns(4,5)P2 enrichment in PRMPs (Fig. 3) above the increase in the general membrane material content agrees well with the reported ~40% higher PtdIns(4,5)P2 concentration in the membranes of phagosomal cups (6). In the latter study, local membrane density was controlled for by myristoylated GFP localized to the plasma membrane, and fluorescence images obtained with a confocal microscope were used for quantitative analysis. These results are also consistent with quantitative electron microscopic studies indicating that PtdIns(4,5)P2 is enriched in the lamellipodia of HEK-293 astrocyes (68). Taken together, converging lines of evidence based on different techniques have confirmed the concept that PtdIns(4,5)P2 can be laterally segregated in micrometer-sized domains in 2D lipid bilayers. Interestingly, both the results of phagocytosis studies and the results derived from Fig. 3 and 4 indicate that such lateral segregation preferentially occurs at the same sites at which local membrane folds concentrate PtdIns(4,5)P2 in 3D space.

**PRMPs colocalize exclusively with regions of dense cortical F actin.** Directing actin polymerization is one of the major and best-characterized signaling functions of PtdIns(4,5)P2 (10, 32, 54, 58). We therefore examined the spatial relationship between PRMPs and the underlying actin cytoskeleton (Fig. 5). In a three-color colocalization study, PtdIns(4,5)P2 localization is revealed by a fusion protein linking ECFP to the PH domain.
of PLCδ1 (ECFP/PLCδ1-PH). ECFP/PLCδ1-PH shows the same binding specificity for PtdIns(4,5)P₂ on the plasma membrane (Fig. 5A and 9A) as EGFP/PLCδ1-PH, and a mutant form of ECFP/PLCδ1-PH deficient in PtdIns(4,5)P₂ binding (i.e., R40L) displays uniform fluorescence distribution in the cytoplasm (data not shown). A fully differentiated adipocyte (i.e., day 7 differentiation) (35) transiently expressing ECFP/PLCδ1-PH at a high level (i.e., intracellular aggregates of the fusion protein indicated by the arrow in panel G) are shown in panels G to I with 8-bit single-color intensity scales (cyan, ECFP/PLCδ1-PH; green, Alx488-ConA; red, RhD-Phd). The cell shown in panels G to I is accessible to RhD-Phd labeling, as indicated by the intracellular RhD fluorescence (arrow in panel I). Adipocytes were serum starved for >2 h, and the cell in panels G to I was subsequently insulin stimulated at 37°C for 10 min. Scale bars, 5 μm.

FIG. 5. PRMPs associate exclusively with regions of dense cortical F-actin. Adipocytes expressing ECFP/PLCδ1-PH (A, D, and G) were fixed and labeled with Alx488-ConA for the plasma membrane (B, E, and H), followed by cell permeabilization and F-actin staining with RhD-Phd (C, F, and I). High-magnification (i.e., 66 nm/pixel) image stacks consisting of 17 z sections were acquired with negligible cross talk among the three fluorescence channels (see Materials and Methods) and were processed with a superresolution deconvolution algorithm (11). Distribution patterns for the fluorescent probes (A to F) were visualized with 8-bit false-color intensity scales and were contrast enhanced at between 10 and 100 for panels A and D, between 10 and 70 for panels B and E, and between 10 and 50 for panels C and F. The cell is viewed both from the top (A to C) and from the side (D to F; the side indicated by the arrow in panel B). In addition, single-plane images of an adipocyte expressing ECFP/PLCδ1-PH at a high level (i.e., intracellular aggregates of the fusion protein indicated by the arrow in panel G) are shown in panels G to I with 8-bit single-color intensity scales (cyan, ECFP/PLCδ1-PH; green, Alx488-ConA; red, RhD-Phd). The cell shown in panels G to I is accessible to RhD-Phd labeling, as indicated by the intracellular RhD fluorescence (arrow in panel I). Adipocytes were serum starved for >2 h, and the cell in panels G to I was subsequently insulin stimulated at 37°C for 10 min. Scale bars, 5 μm.
the cortical cytoskeleton. This hypothesis is consistent with the observation that drastic overexpression of ECFP/PLC61-PH, as indicated by the intracellular aggregation of the fusion protein (arrow in Fig. 5G), prevents cortical F-actin formation in both serum-starved cells (data not shown) and insulin-stimulated cells (Fig. 5G to I, 10, and 11). That this effect is specific is confirmed by the fact that Alexa488-ConA staining of the outer leaflet of the plasma membrane is not visibly affected (Fig. 5H). In contrast, cortical F-actin formation relies on the direct accessibility of PtdIns(4,5)P2 on the inner leaflet of the plasma membrane, which is blocked by the overexpression of ECFP/PLC61-PH (Fig. 5I).

The exclusive colocalization between PRMPs and regions of dense F-actin (Fig. 5) prompted us to address whether the plasma membrane localization of PtdIns(4,5)P2 and PRMP formation are dependent on actin polymerization. It was previously reported (35) and confirmed in the present studies that a layer of cortical F-actin is formed in fully differentiated 3T3-L1 adipocytes. For example, at day 7 of differentiation, 82% ± 5% (Fig. 6A) of cultured adipocytes possess distinct cortical F-actin upon Rho-Phd staining (Fig. 5A to F). Incubation of these cells with the actin polymerization inhibitor LB severely disrupts F-actin formation (Fig. 6A). However, in 32% ± 4% of these LB-treated cells, some of the F-actin cytoskeleton is preserved. The presence of cortical F-actin in these fully differentiated adipocytes and its disruption by LB are not dependent on acute insulin stimulation (35), although insulin can enhance the formation of additional F-actin in serum-starved adipocytes (33). In a population (i.e., ~18%) of unstimulated adipocytes that do not show distinct cortical F-actin, we found that all adipocytes expressing EGFP/PLC61-PH have clear plasma membrane localization of the fusion protein (Fig. 6B). Thus, without LB treatment, display of PtdIns(4,5)P2 on the plasma membrane is independent of insulin stimulation and the cortical cytoskeleton. In LB-treated cells, 100% ± 1% adipocytes with at least some cortical F-actin display plasma membrane localization of ECFP/PLC61-PH, and the percentage decreases to 78% ± 2% in cells that are completely devoid of F-actin (Fig. 6B). This result is likely due to the potential toxicity of LB for a kinase(s) that synthesizes PtdIns(4,5)P2 in cells that take up large amounts of this drug. Overall, however, PtdIns(4,5)P2 localization to the plasma membrane is largely independent of cortical F-actin formation.

High-magnification optical sections of four LB-treated adipocytes expressing EGFP/PLC61-PH but completely devoid of F-actin were acquired and deconvolved with the superresolution deconvolution algorithm. PRMPs still form in these cells (see movie 5 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) and are qualitatively indistinguishable (in three out of four cells) from those observed in control live or fixed cells (Fig. 1, 2, 5, 7, 12, and 13). Live adipocytes that did not undergo the electroporation process were also labeled with Alexa594-ConA. These cells were quickly fixed and examined with the superresolution deconvolution imaging technique. An elaborate PRMP-like network is found on the surface of these cells (see movie 6 at http://invitro.umassmed.edu/~sh/supplements/supplements.html), indicating that PRMPs are not artifacts of EGF/PLC61-PH expression and/or the electroporation process. Thus, both PtdIns(4,5)P2 localization to the plasma membrane and PRMP formation are independent of cortical F-actin formation. Instead, uneven distribution of plasma membrane content is an intrinsic property of 3T3-L1 adipocytes and other cell lines (see Discussion) and is the major mechanism contributing to PRMP formation (Fig. 2 to 4).

PtdIns(4,5)P2-containing vesicular structures beneath PRMPs. It has long been established that PtdIns(4,5)P2 plays a crucial role in clathrin-assisted endocytosis by recruiting multiple molecular components to the plasma membrane (14, 22, 43). Subsequent PtdIns(4,5)P2 hydrolysis on the nascent endo-

FIG. 6. Display of PtdIns(4,5)P2 in the plasma membrane of 3T3-L1 adipocytes is independent of the cortical F-actin cytoskeleton. Morpherologically differentiated adipocytes (day 7) expressing EGFP/PLC61-PH were serum starved for >2 h. A portion of the cells was incubated with 60 μM LB for 2 h at 37°C. The cells were fixed in 4% formaldehyde, followed by Rh-Phd staining for F-actin (Fig. 5). (A) Percentages of cells displaying at least partial cortical F-actin (i.e., F-actin positive) with (w/) or without (w/o) LB treatment. PtdIns(4,5)P2 production was monitored by the plasma membrane localization of EGFP/PLC61-PH. (B) Percentages of cells with plasma membrane (Memb.) PtdIns(4,5)P2 (PIP2) as a function of LB treatment and intactness of cortical F-actin. Error bars are standard errors of the mean from two independent experiments. [See movie 5 at http://invitro.umassmed.edu/~sh/supplements/supplements.html for the distribution of PtdIns(4,5)P2 on the surfaces of two adipocytes completely devoid of F-actin.]
FIG. 7. PtdIns(4,5)P₂-containing vesicular structures just beneath the PRMP of a fixed adipocyte. (A, B, and E) Two adipocytes transiently expressing EGFP/PLCᵦ₁-PH (A and E) were labeled with Alx594-ConA (B) after cell fixation. The dynamic range of the deconvolved 3D image obtained with the EGFP fluorescence channel (E) was mapped to an 8-bit false-color intensity scale and contrast enhanced at between 10 and 100. The images in panels A and B are the 17th z sections of their respective 3D deconvolved images. The dimensions of the 3D grid in panel E are
osomes is believed to be important for the clathrin coat disassembly that precedes endosome maturation toward the interior of a cell or recycling back to the plasma membrane (22). However, this dynamic process of PtdIns(4,5)P₂ turnover has not been directly observed. We hypothesized that the PtdIns(4,5)P₂-containing structures found beneath the plasma membrane (e.g., peak 4 in Fig. 4) are related to adipocyte endocytic activities. Therefore, these structures were examined in further detail by superresolution deconvolution microscopy in Fig. 7 to 9.

Figure 7 shows two adjoining adipocytes, both expressing EGFP/PLC₁δ₁-PH (Fig. 7A) and labeled with Alexa594-ConA (Fig. 7B) after cell fixation. Intensity profiles for lines drawn across the two cells or the right-side cell alone are plotted in Fig. 7C and D, respectively (green line, EGFP/PLC₁δ₁-PH labeling; red line, Alexa594-ConA staining). Because of the resolution limit (i.e., >200 nm) of optical microscopy, the center plasma membranes attaching the two cells appear as a single fluorescence entity (Fig. 7A to C). As expected, the fluorescence intensities associated with these center membranes are approximately double those of the single plasma membranes on the other sides of these cells (Fig. 7C). This result indicates that Alexa594-ConA is a sensitive indicator of local membrane density and that the local PtdIns(4,5)P₂ concentration is approximately proportional to the total lipid content in these membrane regions. In contrast, the right-side cell appears to be polarized, with one side of the plasma membrane being enriched in both PtdIns(4,5)P₂ and overall lipid contents (i.e., a PRMP) (red arrows in Fig. 7). Furthermore, the PRMP, when visualized with EGFP/PLC₁δ₁-PH fluorescence (Fig. 7A), appears to be thicker than its Alexa594-ConA-labeled counterpart (Fig. 7B). Correspondingly, there is an additional shoulder associated with the EGFP/PLC₁δ₁-PH peak (arrow in Fig. 7D) at ~1 μm beneath the cell boundary defined by both EGFP/PLC₁δ₁-PH and Alexa594-ConA labeling (left peaks in Fig. 7D).

To resolve these additional PtdIns(4,5)P₂-containing structures, superresolution deconvolution images were acquired at a lateral resolution of ~100 nm (Fig. 7E and F) (11). The deconvolved 3D image in Fig. 7E (EGFP fluorescence only) shows that the PRMP extends throughout one side of the cell (red arrow). Furthermore, in the colocalization image (Fig. 7F; colocalized regions are white), a magnified view of a 1.5-μm slice of that PRMP (yellow outline in Fig. 7E), shows vesicle-like, PtdIns(4,5)P₂-rich structures (green) just beneath the plasma membrane defined by extracellular Alexa594-ConA staining (red). Within the resolution of the microscopic technique, these “vesicles” are ~100 to 300 nm in diameter and appear to be interconnected and partially attached to the plasma membrane (blue arrow in Fig. 7F). In contrast, no similar PtdIns(4,5)P₂-containing membranes are found further inside the cells (Fig. 7). This proximity of PtdIns(4,5)P₂-containing “vesicles” to PRMPs is found in three out of four adipocytes examined in the superresolution fashion, suggesting that PRMPs may spatially organize endocytic activities (see below).

The dynamic nature of these PtdIns(4,5)P₂-containing “vesicles” is revealed by differential Alexa594-ConA staining before (Fig. 5B to D) and after (Fig. 8A) adipocyte fixation. Because surface Alexa594-ConA is continuously internalized, various stages of the endocytosis process can be immobilized in fixed adipocytes so that structural details can be resolved later by superresolution deconvolution microscopy. The difference in Alexa594-ConA staining of living and fixed adipocytes is most apparent for the bottom membranes attached to coverslips (0.75-μm z sections) (Fig. 8A and B). When Alexa594-ConA (red regions in Fig. 8B) is applied to living adipocytes at 37°C, the probe is able to label lipid-rich structures on the bottom membrane together with EGFP/PLC₁δ₁-PH (green regions in Fig. 8B). On the other hand, Alexa594-ConA cannot diffuse to the bottom membrane after cell fixation, and only EGFP/PLC₁δ₁-PH is present there (Fig. 8A). When live cells are stained, the process of Alexa594-ConA internalization appears to be manifested initially as endocytic vesicles containing both EGFP/PLC₁δ₁-PH and Alexa594-ConA (Fig. 8C; colocalized regions are white) and lining the inner surface of the plasma membrane (green arrows in Fig. 8C). It also appears that, when these endocytic membranes mature toward the interior of the cell, the Alexa594-ConA label progressively dominates (green arrows in Fig. 8C). Importantly, only Alexa594-ConA persists on the intracellular vesicles (cyan arrowheads in Fig. 8C). This apparent maturation process is most obvious in a magnified region (i.e., a 1-μm-thick z section) of another adipocyte showing massive endocytic activities, where “vesicles” containing only Alexa594-ConA appear to emerge from the tip of this membrane trafficking event while “vesicles” still containing EGFP/PLC₁δ₁-PH are closer to and partially attached to the plasma membrane (Fig. 8D). Taken together, the data presented in Fig. 7 and 8 are consistent with the hypothesis that PtdIns(4,5)P₂ is lost from endocytic membranes early in the endocytosis process (15, 22).

**PRMPs colocalize with active zones of clathrin-assisted endocytosis.** To establish a direct link between the PtdIns(4,5)P₂-containing “vesicles” and clathrin-assisted endocytosis, three-color colocalization experiments were carried out to determine the spatial relationship among PRMPs, clathrin heavy chains, and membrane density in cultured adipocytes (Fig. 9). In these studies, PtdIns(4,5)P₂ is targeted with ECFP/PLC₁δ₁-PH (blue regions in Fig. 9A). The plasma membrane is distinct from the endocytic membrane, as determined by extracellular Alexa488-ConA labeling after cell fixation (green regions in Fig. 9B; see also discussion about Fig. 8). The cell was subsequently deter-
gent permeabilized, and clathrin heavy chains were immunofluorescently labeled with RhD-derivatized antibody (red regions in Fig. 9C). As expected, clathrin heavy chains concentrate in the perinuclear region and on the plasma membrane (Fig. 9C), where clathrin-assisted membrane trafficking predominantly occurs (43). As a result of detergent permeabilization, Alx488-ConA probe also leaks into the cell (Fig. 9B). Despite this complication, it is clear from examining the 3D projection
images that the PtdIns(4,5)P$_2$- and membrane-rich region (i.e., a PRMP) (arrows in Fig. 9A and B) also contains increased amounts of the clathrin subunit (arrow in Fig. 9C). Whether or not the cells are insulin stimulated (Fig. 9) or serum starved (data not shown), this spatial correlation between active regions of clathrin-assisted endocytosis and PRMPs are observed in all nine adipocytes examined by the three-color colocalization method. These results are consistent with the hypothesis that PtdIns(4,5)P$_2$ enrichment in PRMPs leads to enhanced PtdIns(4,5)P$_2$ interactions with the downstream effector molecules that assemble the clathrin coat (22, 43), thus focusing endocytic activities in active zones of the adipocyte surface membrane.

The detailed spatial relationships identified for the cellular structures by the three fluorescent labels are revealed upon examination of magnified colocalization images (sample $z$ sections of 0.5 $\mu$m) (Fig. 9D to F). Figure 9D shows extensive colocalization (white regions) between clathrin heavy chains (red regions) and PtdIns(4,5)P$_2$-containing membranes (blue regions). Some PtdIns(4,5)P$_2$-containing structures are distinct from the plasma membrane, which is marked with extracellular Alx488-ConA labeling (green regions in Fig. 9E). Since clathrin-coated endocytotic vesicles originate from the inner leaflet of the plasma membrane, while Alx488-ConA labels the outer leaflet, there is only a partial overlap in anticlathrin and Alx488-ConA fluorescence (Fig. 9F), with these two signals being biased toward the interior and toward the exterior of the cell, respectively. Detailed examination of Fig. 9D to F further reveals PtdIns(4,5)P$_2$-containing and clathrin-coated “vesicles” emerging from the plasma membrane (yellow arrowheads). There are also nascent “vesicles” predominantly decorated with clathrin heavy chains and containing little or no PtdIns(4,5)P$_2$ (yellow arrows). Similarly, a few PtdIns(4,5)
FIG. 10. Transferrin endocytosis in 3T3-L1 adipocytes. (A, C, E, and H) Differentiated adipocytes (day 5) expressing hTFRc were incubated with Alexa488-TF for 1 h at 4°C to allow ligand-receptor binding on the plasma membrane (arrow in panel A). The cells were subsequently warmed to 37°C and incubated for 15 min to allow transferrin uptake (arrows in panels C and E). (B, D, F, and I) The cells were fixed in 4% formaldehyde and stained with Rho-PhD for F-actin. Arrows in panels B and D indicate cortical F-actin, while actin filaments were disrupted by either LB treatment (F) or expression of ECFP/PLCδ1-PH (I). (G and H) Expression of the fusion protein (G) sequesters plasma membrane PtdIns(4,5)P₂ but does not interfere with Alexa488-TF binding to its receptors on the cell surface (H). However, transferrin uptake in these cells is inhibited (H). In contrast, a majority of cells in which cortical F-actin is completely disrupted by LB treatment have normal transferrin endocytosis (E and F). Interestingly, transferrin labeling of the cell surface appears to be polarized [arrows in panels G and H indicate a membrane region enriched in PtdIns(4,5)P₂, and surface-bound transferrin]. Alexa488 fluorescence in panel H was imaged in the YFP channel to avoid fluorescence from ECFP/PLCδ1-PH (see Materials and Methods). Scale bars, 5 μm.
P2-containing “vesicles” with residual clathrin labeling are found close to the plasma membrane (cyan arrowheads). Importantly, colocalization between clathrin heavy chains and PtdIns(4,5)P2 in these images (Fig. 9D to F) occurs only in the vicinity of the plasma membrane. These observations provide direct evidence supporting the hypothesized PtdIns(4,5)P2 turnover during clathrin-assisted endocytosis (22, 43). Slice-by-slice colocalization images of the PRMP shown in Fig. 9A to C are shown in movie 7 at http://invitro.umassmed.edu/~sh/supplements.html.

PtdIns(4,5)P2 but not F actin is critically important for transferrin endocytosis. The concomitant enrichment of PtdIns(4,5)P2 and cortical F actin within and beneath PRMPs, respectively (Fig. 2 to 5), suggests that both may play roles in clathrin-assisted endocytosis. Since receptor-mediated transferrin uptake is a classic example of clathrin-assisted endocytosis, this process was investigated in 3T3-L1 adipocytes as a function of PtdIns(4,5)P2 availability and actin polymerization (Fig. 10 and 11). Differentiated adipocytes expressing hTFRc were incubated with Alexa488-conjugated transferrin at 4°C to allow ligand-receptor binding on the plasma membrane (arrow in Fig. 10A). The cells were subsequently warmed to 37°C and incubated for 15 min to facilitate transferrin uptake (arrows in Fig. 10C and E). Some of these cells also highly express ECFP/PLCδ1-PH (Fig. 10G), which sequesters PtdIns(4,5)P2 in the plasma membrane and in some cases completely prevents cortical F-actin formation (Fig. 5G to I and 10G to I). Alternatively, F-actin was disrupted by LB treatment (Fig. 10E and F). As visualized by RhD-PhD staining, 77% ± 4% of the hTFRc-expressing adipocytes have cortical F-actin (Fig. 11A) (35). This percentage decreases to 41% ± 6.7% for cells expressing EGFP/PLCδ1-PH and to 25% ± 5% for LB-treated cells (Fig. 11A). For cells that have cortical F-actin, 93% ± 2% show clear transferrin endocytosis after 15 min (Fig. 10C and D). This percentage decreases to 34% ± 1% for cells expressing EGFP/PLCδ1-PH but retaining cortical F-actin (Fig. 11B). For cells expressing the fusion protein and completely lacking F-actin, only 10% ± 2% undergo transferrin endocytosis. This further inhibition in transferrin endocytosis (i.e., from 34% ± 1% to 10% ± 2%) in EGFP/PLCδ1-PH expressing cells is only partially attributed to the loss of the cortical cytoskeleton, since disruption of F-actin with LB causes only a slight decrease in the rate of transferrin uptake (i.e., from 93% ± 2% to 84% ± 4%) (Fig. 11B). Rather, in cells that lack cortical F-actin due to high-level EGFP/PLCδ1-PH expression, PtdIns(4,5)P2 in the plasma membrane likely is sequestered to a higher degree and therefore is more effective in inhibiting transferrin endocytosis than cortical F-actin. Thus, it appears that clathrin-assisted endocytosis is critically dependent on the

**FIG. 11.** Transferrin endocytosis is critically dependent on plasma membrane PtdIns(4,5)P2 but is only modestly facilitated by the actin cytoskeleton. (A) Summary of cortical F-actin formation (i.e., F-actin positive) as a function of ECFP/PLCδ1-PH expression (i.e., PLCδ1-PH positive) or LB treatment. Cells in which a complete or a partial cortical actin cytoskeleton was preserved were counted as F-actin positive. (B) Percentages of cells undergoing transferrin endocytosis as a function of plasma membrane PtdIns(4,5)P2 (sequestered by ECFP/PLCδ1-PH) and/or F-actin (disrupted by LB treatment or ECFP/PLCδ1-PH expression). Error bars are standard errors of the mean from two independent experiments.
availability of PtdIns(4,5)P$_2$ in the plasma membrane but is only partially facilitated by the cortical F-actin cytoskeleton.

**PRMPs appear to direct Myo1c-induced membrane ruffling near the substratum.** Based on data showing that cortical actin filaments are concentrated beneath PRMPs in cultured adipocytes (Fig. 5), we tested whether actin-based functions may be similarly localized at PRMP sites. One actin-based cellular process is plasma membrane ruffling (27, 45), which has been reported to be intensified by insulin stimulation in 3T3-L1 adipocytes (33, 35). However, ruffling events occur mainly at the adherent membrane of adipocytes in cultures (35) and are fairly infrequent. However, the frequency and size of plasma membrane ruffles in cultured adipocytes can be amplified by the expression of unconventional Myo1c (5). We therefore first evaluated the distribution of expressed Myo1c in 3T3-L1 adipocytes. Figure 12 shows that a representative adipocyte transiently expressing both ECFP/PLC$_{81}$-PH (Fig. 12A) and a fusion protein linking EYFP to Myo1c (EYFP/Myo1c) (Fig. 12B) displays PRMPs colocalized exclusively with regions of concentrated EYFP/Myo1c. Furthermore, in the colocalization image (Fig. 12C; red regions indicate ECFP/PLC$_{81}$-PH, green regions indicate EYFP/Myo1c, and white regions indicate colocalization), EYFP/Myo1c fluorescence is localized mostly beneath the inner leaflet of the plasma membrane labeled with ECFP/PLC$_{81}$-PH. Thus, EYFP/Myo1c is concentrated at regions of dense F-actin, which are also exclusively localized at PRMPs (Fig. 5). These findings are consistent with electron microscopic images showing that Myo1c is localized mostly at the cortical F-actin network beneath the plasma membrane of cultured adipocytes (4).

In live cells, the overexpression of Myo1c leads to dramatic ruffling events near the substratum (Fig. 13; see also movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) (5). Large membrane ruffles have also been observed, albeit at a much lower frequency, in normal adipocytes (yellow arrows in Fig. 1D). The fact that these ruffles occur predominantly in membranes close to the coverslip (Fig. 1D and 13; see also movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) is consistent with current hypotheses on actin-based cellular motility (45). Thus, a membrane protrusion occurring at the bottom of a cell, possibly driven by concentrated Myo1c activity on F-actin, can briefly attach to the coverslip. Since the immobile adipocyte does not retract on the opposite end of this protrusion event, this temporary attachment cannot be sustained. Therefore, the membrane protrusion eventually detaches and peels back, resulting in a classic ruffling event (see movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). In contrast, there are no physical structures for membrane attachment in the spaces above the coverslip, and large-scale membrane ruffles are seldom observed in these regions in our studies. Importantly, PRMPs are found immediately above the Myo1c-induced membrane ruffles (labeled 1 to 3 in Fig. 13A), and little large-scale ruffling occurs in regions not associated with PRMPs.
Detailed examination of movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html reveals that the PRMP above the most active ruffle (ruffle 1) persists throughout the experiment, while the one above the least active ruffle (ruffle 2) diminishes (Fig. 13). Significantly, all PRMPs regroup somewhat at these same membrane sites toward the end of the experiment (Fig. 13F), suggesting a localized mechanism coordinating PtdIns(4,5)P₂ concentration in the plasma membrane, F-actin dynamics, and Myo1c-driven membrane trafficking at these specialized membrane patches.
DISCUSSION

An important conclusion derived from the present findings is that plasma membranes of cultured adipocytes are complex and heterogeneous structures that contain organized zones of high actin polymerization, endocytic, and membrane-ruffling activities. Using advanced imaging techniques, we found that both lipid and protein constituents of the plasma membrane are distributed nonuniformly in 3D space. This distribution has the significant consequence of concentrating the signaling molecule PtdIns(4,5)P₂ in micrometer-sized plasma membrane patches (i.e., PRMPs) (Fig. 1 to 5, 7 to 9, 12, and 13). Such preferential concentration of PtdIns(4,5)P₂ in specific regions of adipocyte surface membranes likely enhances PtdIns(4,5)P₂ interactions with downstream effectors [e.g., PtdIns(4,5)P₂-binding components of protein complexes that regulate actin polymerization and endocytosis]. Indeed, PRMPs are involved in the spatial organization of cortical F-actin (Fig. 5) and colocalize with active zones of endocytosis (Fig. 7 to 9). PRMPs display little movement on the cell surface of cultured adipocytes (see movie 4 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). However, in adipocytes whose motility is dramatically enhanced by the expression of Myo1c, PRMPs show profound spatial correlation with Myo1c-induced membranes ruffles (Fig. 12 and 13; see also movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). Thus, the imaging data presented here support the hypothesis that PRMPs constitute the major structural basis upon which multiple PtdIns(4,5)P₂-directed cellular activities take place. Interestingly, insulin stimulation does not seem to alter the global organization of PRMPs (see movie 4 at http://invitro.umassmed.edu/~sh/supplements/supplements.html).

The fusion protein EGFP/PLC61-PH has been demonstrated in many laboratories to be a sensitive indicator of PtdIns(4,5)P₂ in multiple cell types (2, 3, 44). In agreement with previous observations (48, 56, 67), PtdIns(4,5)P₂ is found primarily in the plasma membrane of cultured 3T3-L1 adipocytes. EGFP/PLC61-PH affinity toward PtdIns(4,5)P₂ is relatively weak (Kₘ, ~2 μM) (39), and the plasma membrane-bound fusion protein undergoes rapid (≈1 s) equilibrium with its cytosolic counterpart (64). Therefore, EGFP/PLC61-PH is specifically targeted to plasma membrane PtdIns(4,5)P₂ but is unlikely to induce lateral PtdIns(4,5)P₂ segregation in micrometer-sized membrane domains (44). Furthermore, a major mechanism contributing to PRMP formation is local membrane folding concentrating PtdIns(4,5)P₂ in 3D space (Fig. 2 to 4). Such an uneven distribution of plasma membrane content is intrinsically present on the adipocyte surface, as evidenced by 3D images (see movie 6 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) of live cells labeled only with Alx594-ConA. Therefore, PRMPs are not the result of EGFP/PLC61-PH expression or the electroporation process introducing plasmid DNA. On the other hand, EGFP/PLC61-PH is not very useful for visualizing PtdIns(4,5)P₂ in intracellular membranes because intracellular PtdIns(4,5)P₂ is difficult to image against the background fluorescence of EGFP/PLC61-PH solubilized in the cytosol or bound to Ins(1,4,5)P₃. In fact, quantitative electron microscopic results have shown that the level of EGFP/PLC61-PH expressed in the cytoplasm is higher than those in intracellular membranes but significantly lower than that on the plasma membrane (68). Nevertheless, under favorable expression and imaging conditions, we observed some EGFP/PLC61-PH localization in the perinuclear region of adipocytes (data not shown), where the fusion protein presumably binds to PtdIns(4,5)P₂ in Golgi membranes (23, 34).

Heterogeneous segregation of PtdIns(4,5)P₂ in plasma membranes was previously reported based on single-plane, wide-field, or confocal microscopy (38, 59, 66). However, the full extent of PtdIns(4,5)P₂ spatial organization is revealed in the present studies by use of a custom-built, laser-illuminated, ultrafast-acquisition deconvolution microscope. This technique allowed us to acquire 3D images within a time frame (≈1 s) that is virtually insensitive to cell movements and with minimal interference from photobleaching (51). During the course of our studies, a report of PtdIns(4,5)P₂-rich membrane domains in another cell type surprisingly suggested that they may result entirely from local membrane folds unresolved by fluorescence microscopy (66). This hypothesis is supported by our observation that PRMPs in adipocytes colocalize with membrane-dense domains labeled with BODIPY-HPC or Alx594-ConA (Fig. 2 to 4). The existence of such PRMPs is consistent with electron microscopic images showing numerous small (i.e., 25 to 150 nm) invaginations and vesicles in and near the surface membranes of rat adipocytes (12, 61). Remarkably, clusters of these suboptical-resolution structures organize into large patches that are apparent on the adipocyte surface under fluorescence microscopic observation (61). These membrane invaginations significantly increase the total surface area of an adipocyte cell and may reflect adaptive specialization of the plasma membrane to handle a massive membrane trafficking volume occurring in this particular cell type (61). Furthermore, quantitative, ratiometric image analysis carried out in the present studies indicates that PtdIns(4,5)P₂ is also laterally concentrated in PRMPs (Fig. 3 and 4). These data agree with previous results obtained from quantitative fluorescence and electron microscopic studies demonstrating PtdIns(4,5)P₂ enrichment in the membranes of phagosomal cups and lamellipodia, respectively (6, 68). On the basis of earlier data and our present results, the mechanisms of increased membrane content and increased lateral PtdIns(4,5)P₂ concentration both appear to contribute to the formation of PRMPs.

Regardless of the specific mechanism for concentrating PtdIns(4,5)P₂ in PRMPs, we found that these plasma membrane structures are effective in concentrating PtdIns(4,5)P₂-directed actin polymerization (Fig. 5) and endocytic (Fig. 7 to 9) activities in segregated regions on the adipocyte surface. The importance of PtdIns(4,5)P₂ in managing F-actin assembly and remodeling has been well documented (10, 32, 54, 58). We found that PRMPs are exclusively associated with regions of dense cortical F-actin (Fig. 5). This direct correlation between the spatial concentrations of PtdIns(4,5)P₂ and F-actin is consistent with atomic force microscopic measurements, which demonstrated that the adhesion forces between the plasma membrane and its underlying F-actin cytoskeleton were directly proportional to PtdIns(4,5)P₂ levels in the membrane (50). In addition, we find that sequestration of PtdIns(4,5)P₂ in the plasma membrane by the overexpression of EGFP/PLC61-PH inhibits cortical F-actin formation (Fig. 5G to I, 10G to I, and 11A). These results indicate that PRMPs play a
role in the spatial organization of the actin cytoskeleton. In contrast, the disruption of cortical F-actin by LB does not interfere with PtdIns(4,5)P$_2$ production or PRMP formation (Fig. 6; see also movie 5 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). It appears that the uneven distribution of plasma membrane content, a major mechanism contributing to PRMP formation (Fig. 2 to 4), is an intrinsic property of animal cells (24). Through the use of various techniques and cell types, it has been estimated that 40 to 1,000% excess membrane is stored in membrane folds, microvilli, and caveolae (17, 19, 52, 55, 61, 72, 73). Many mechanisms could contribute to such complexity in the 3D organization of the plasma membrane; these include lipid compositions (8, 29), membrane-protein interactions (20, 44), and cytoskeletal elements (spectrin-ankyrin network, F-actin, microtubules, intermediate filaments, and so forth) (24, 31). These mechanisms cannot be completely abolished by the disruption of F-actin alone (see movie 5 at http://invitro.umassmed.edu/~sh/supplements/supplements.html), consistent with the fact that adipocytes completely lacking cortical F-actin still maintain a distinct irregular cellular morphology characteristic of fully differentiated adipocytes (see movie 5 at http://invitro.umassmed.edu/~sh/supplements/supplements.html).

Increasing evidence indicates that PtdIns(4,5)P$_2$ functions in highly localized membrane structures to regulate cellular processes (14, 15, 32, 43, 44, 48). Studies on large-scale membrane dynamics associated with phagocytosis and bacterial invasion have demonstrated that transient, focal PtdIns(4,5)P$_2$ accumulation is essential for developing membrane protrusions surrounding the invading particles. Interestingly, in those previous studies, the complete loss of PtdIns(4,5)P$_2$ on internalized membranes coincided with membrane fission (6, 60). Consistent with these findings, our results obtained with superresolution deconvolution microscopy reveal PtdIns(4,5)P$_2$-containing, submicrometer-sized vesicular structures immediately beneath PRMPs (Fig. 7). PtdIns(4,5)P$_2$ is subsequently eliminated from such “vesicles” maturing toward the interior of the cell (Fig. 8C and D). These observations suggest that PtdIns(4,5)P$_2$ dynamics associated with phagocytosis and bacterial invasion may also occur on smaller spatial scales and are associated with clathrin-assisted endocytosis (15, 43). Clathrin coat assembly on budding endosomes is thought to depend on the local presence of PtdIns(4,5)P$_2$ while PtdIns(4,5)P$_2$ hydrolysis precedes clathrin coat disassembly and probably also endosome fission (15, 22). This hypothesis is directly supported by the images shown in Fig. 9. In a membrane region associated with active endocytosis, budding “vesicles” containing both clathrin heavy chains and PtdIns(4,5)P$_2$ are found to emerge from the inner leaflet of the plasma membrane (Fig. 9D to F; see also movie 7 at http://invitro.umassmed.edu/~sh/supplements/supplements.html). Most significantly, a PMPR completely colocalizes with this active region of endocytosis (Fig. 9A to C), consistent with the observation that plasma membrane PtdIns(4,5)P$_2$ is critically important for transferrin uptake, a finding which adds to a large body of conflicting evidence relating to the exact function of F-actin in the endocytosis process (18, 21, 49). The correlations between the spatial dispositions of PRMPs in the adipocyte plasma membrane and similarly localized zones of concentrated F-actin and high endocytic activities do not provide conclusive evidence that there is a causative link between them. It could be argued that more endocytosis occurs at PRMPs simply due to the larger amounts of membranes present at these regions to be internalized. In order to further investigate the issue of the functionality of PRMPs, we used a protein, the molecular motor Myo1c, which is known to require F-actin for its actions (4). As expected, expressed Myo1c localized predominantly at regions of high cortical F-actin levels that occur just beneath PRMPs (Fig. 12). Significantly, the membrane ruffling that is mediated by Myo1c in these adipocytes occurs predominantly at substrate locations marked by PRMPs (Fig. 13; see also movie 8 at http://invitro.umassmed.edu/~sh/supplements/supplements.html) (5). In other words, Myo1c apparently can function efficiently to cause large membrane ruffles only at sites on the plasma membrane where it is concentrated by large amounts of F-actin, beneath PRMPs. These data indicate that a function of PRMPs is to localize membrane-ruffling events, presumably by concentrating F-actin and Myo1c to enhance their promotion of ruffling. Little or no membrane ruffling occurs away from PRMPs in the adipocyte plasma membrane. These considerations provide strong support for the hypothesis that PRMPs have important functions in directing cellular processes in adipocytes. The fact that Myo1c also promotes GLUT4 translocation in response to insulin (4) suggests that PRMPs may also promote this process. Future experiments are directed toward testing this possibility.

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