COPI buds 60-nm lipid droplets from reconstituted water–phospholipid–triacylglyceride interfaces, suggesting a tension clamp function

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Intracellular trafficking between organelles is achieved by coat protein complexes, coat protoomers, that bud vesicles from bilayer membranes. Lipid droplets are protected by a monolayer and thus seem unsuitable targets for coatomers. Unexpectedly, coat protein complex I (COPI) is required for lipid droplet targeting of some proteins, suggesting a possible direct interaction between COPI and lipid droplets. Here, we find that COPI coat components can bud 60-nm triacylglycerol nanodroplets from artificial lipid droplet (LD) interfaces. This budding decreases phospholipid packing of the monolayer decorating the mother LD. As a result, hydrophobic triacylglycerol molecules become more exposed to the aqueous environment, increasing LD surface tension. In vivo, this surface tension increase may prime lipid droplets for reactions with neighboring proteins or membranes. It provides a mechanism fundamentally different from transport vesicle formation by COPI, likely responsible for the diverse lipid droplet phenotypes associated with depletion of COPI subunits.

We tested Arf1 binding to LDs with two complementary approaches: flotation assay and microfluidics. We prepared TAG droplets that were surrounded by a monolayer of a phospholipid mixture (PL) of the same composition as that used to prepare control liposomes (PL composition is similar to that of natural LDs) (18). Arf1 binds to such droplets in a GTP-dependent manner and with a similar efficiency as on liposomes (Fig. 14). We confirmed Arf1 binding to buffer/TAG interfaces using a microfluidic setup allowing direct visualization of protein interactions. We produced micrometric buffer droplets in a stream of oil containing the phospholipids. The buffer/TAG interface is then coated with a monolayer of PL, as attested by the change in surface tension (see Fig. 4). In each buffer drop, biochemical reactions taking place at the buffer/TAG interface can be observed by fluorescence microscopy. The small buffer volume minimizes the amount of coatomer and Arf1 required, a decisive advantage compared with the inverse geometry where oil droplets are produced in a stream of buffer. Fig. 1B shows images of buffer droplets containing Cy3-labeled Arf1 and, alternatively, GDP or GTP. In agreement with the biochemical assay, Cy3–Arf1 accumulates in a GTP-dependent manner at the TAG/buffer interface decorated with a monolayer of PL, confirming that Arf1 is able to bind to the LD lipid monolayer surface.

COPI Machinery Buds Particles from TAG/Buffer Interface. Next, we tested the ability of coatomer to be recruited to Arf1-decorated LDs. We added Alexa 647-labeled coatomer to Cy3–Arfl and GTP to the buffer-in-oil drops. Under these conditions, the fluorescent proteins did not only cover the TAG/buffer interface.
Fig. 1. GTP-specific binding of Arf1 to LDs. (A) Arf1 binds LDs or liposomes with the same efficiency and in a GTP-dependent manner. Extruded liposomes and TAG droplets containing the same amount of exposed PL (0.5 mM) were incubated during 30 min with Arf1-GDP (500 nM) and, when indicated, with EDTA (2 mM) and GTP (100 μM) to promote activation of Arf1. After separation of the free proteins from the liposomes or TAG droplets in a sucrose gradient, the amount of Arf1 bound to the membrane was revealed by SDS-PAGE gel stained with SYPRO Orange staining. The amount of Arf1 bound to liposomes or TAG droplets is similar (lanes 2 and 4). No binding is observed in the absence of GTP (lanes 1 and 3). Lane 5 represents the amount of Arf1 input in the solutions. (B) Specific binding of Arf1 to TAG/buffer interface in buffer drops watched in epifluorescence imaging. In a microfluidics channel (Upper), micrometric buffer droplets are produced in a stream of oil containing the phospholipids. Consecutive buffer drops containing fluorescent Arf1-Cy3 (30 nM), ARNO (200 nM), and alternately GTP (50 μM) or nucleotide-free control are produced with this setup. Arf1 only labels the aqueous/TAG interface when GTP is present (center buffer drop). Limited labeling of the contour is observed in the control drops (outer buffer drops). The Arf1-Cy3 signal looks lower at the interface between buffer drops because they are adherent and the interface is not vertical, which lowers the integrated intensity. (Scale bar, 50 μm.)

Instead, Arf1 and coatomer formed mobile spots in the aqueous volume and at the buffer/TAG interface (Fig. 2A and Movie S1) in a GTP-dependent manner (Fig. 2B). To determine the content of these spots, we performed the experiment with unlabeled Arf1, labeled coatomer, and oil containing a fluorescent dye (Bodipy). Again, colocalized spots were observed (Fig. 2C), suggesting a budding process in which small oil droplets are detached from the buffer/TAG interface by the COPI coat. After addition of ArfGAP3, which promotes GTP hydrolysis of Arf1 and reverses the coating process (19), the coat dissociated from the spots particles (Fig. 2D).

Budded Particles Are 60-nm COPI-Coated LDs. To determine the characteristics of the newly formed particles, we isolated them from the buffer drops (Fig. S1) and observed them by EM. The spots can clearly be identified as nano LDs with coat polymer visible at their surface (Fig. 3A, white arrows and Inset). The coat polymer disappears after treatment with ArfGAP3 (Fig. 3B). The size distribution of these nanodroplets shows that they are monodisperse with a typical diameter of 60 ± 15 nm (Fig. 3C). This distribution is consistent with the estimated size obtained from the diffusion coefficients measured by fluorescence cross-correlation spectroscopy (~90 nm, Fig. S2A) and by independent direct tracking of the particles collected from the buffer drops (~100 nm, Fig. S2B). Taken together, these results show that the COPI machinery is able to function on LDs in the same manner as on lipid bilayers by inducing the budding and fission of 60-nm TAG nanodroplets very close to the size of COPI vesicles (2, 3).

COPI Budding Exclusively Occurs at Interfaces with Low Tension. Because natural LDs undergo shrinking and growth phases, we decided to probe the effect of the state of the LD surface on the efficiency of the COPI-induced budding process. We performed microfluidic experiments with increasing amounts of PL (Fig. S3). As shown in Fig. 4A, the number of COPI-induced nanodroplets formed in the buffer drops dramatically increases between 0.1% and 1% PL per TAG (wt/wt), suggesting that the COPI-coat machinery acts preferentially on a packed PL monolayer. When covering the interface, PLs, thanks to their amphiphilic nature, decrease the surface tension by shielding TAG molecules from the aqueous buffer. Because an interface with a low surface tension is more deformable, a packed PL monolayer should facilitate the budding of COPI nano LDs (20). Following this hypothesis, we used a micromanipulation approach (Fig. S4) to measure the surface tension of LDs at various PL concentrations. Strikingly, the surface tension decreased sharply from ~20 mN/m to a vanishing surface tension (below 0.5 mN/m, the detection limit of the technique) exactly in the range of PL concentration (0.1–1% wt/wt PL/TAG) at which COPI reaches its optimum budding efficiency (Fig. 4A). The COPI machinery acts mainly at low surface tension, below a threshold of 2 mN/m.

Budding Nanodroplets Increases the Surface Tension. The main result of nanodroplet formation should be a decrease in PL packing on the mother LD as nanodroplets “capture” relatively more surface than volume from the mother LD (Fig. S5). Put differently, nanodroplet budding consumes the PL monolayer covering the surface of the mother LD, inducing a decrease in the PL/TAG ratio. Energetically, this consumption will increase the surface tension of the mother LD. With a Langmuir trough we measured the variation of PL packing with the surface tension (and therefore PL/TAG ratio, Fig. S6). Remarkably, a 10% decrease in the PL packing from the maximum pressure (corresponding to maximum lipid packing for which the surface tension is below the detection, 0.5 mN/m) is sufficient to raise the surface tension above the 2 mN/m threshold for COPI budding of nanodroplets. A 10% decrease in the PL packing from the maximum density is achieved by budding off approximately five nanodroplets from a 500-nm mother LD (Fig. S7), a typical size of physiological LDs. Hence, a very limited action of COPI is sufficient to induce a substantial change in surface tension.

LDs with High Surface Tension Can Sense Their Environment. Upon surface tension increase induced by COPI, a mother LD will probably become more prone to react with its environment (e.g., with soluble proteins or membranes). First, we used α-synuclein as a model of soluble proteins, because it is known to bind natural LDs (21) and is able to sense phospholipid packing on bilayers (22). We found that binding of α-synuclein to LDs is highly dependent on the PL packing at the interface. When the monolayer is not fully packed α-synuclein binds to the LD surface, whereas at full PL coverage no binding occurs (Fig. 4B). Second, we tested the ability of LDs of various PL compositions to sense α-synuclein binding to the LD surface.
Colocalization of coat ArfGAP3 is added in two of the samples at different concentrations (50 and 10 nM) corresponding to fractions equal to 0.5 and 0.1 of the Arf1 concentration. The sample, recovered as shown in Fig. S1, is split in three vials. The amount of particles is quantified as described in Fig. S3. ArfGAP3 is added in two of the samples at different concentrations (50 and 10 nM) corresponding to fractions equal to 0.5 and 0.1 of the Arf1 concentration. Colocalization of coat-Alexa 647 and TAG-Bodipy is lost over time compared with the control.

Fig. 2. COPI produces nanodroplets from LDs. (A) Particles containing Arf1 and coatamer appear in the buffer drops in the presence of the COPI machinery (Left is a full image of a buffer drop; the other three panels are large magnifications to better see the particles). Less than 2 min after making the buffer drops containing Arf1–Cy3 (30 nM), coatamer (15 nM) labeled with Alexa 647, GTP (50 μM), and ARNO (200 nM), homogenous Arf1–Cy3 and coat–Alexa 647 spots appear in the aqueous volume and at the buffer/TAG interface. Arf1 (green) and coat (red) spots are colocalized, moving together in the buffer drop (Movie S1). The spots are slightly separated because of the time delay to switch laser in the setup. (Scale bar, 5 μm.) (B) The formation of particles is GTP-dependent. In the controls without GTP, coatamer or Arf1, the amount of spots per area is significantly reduced compared with the experiment with 50 μM GTP, 30 nM Arf1, and 15 nM coatamer (Left). (C) The particles are TAG nanodroplets. Same experiment as in A with unlabeled Arf1 (100 nM) and Bodipy dye (1% wt/wt) in the TAG. After collection of the buffer drops as indicated in Fig. S1, colocalized Bodipy/Alexa 647 spots are observed. (Scale bars, 10 μm.) (D) Loss of colocalization over time after ArfGAP3 addition. The sample, recovered as shown in Fig. S1, is split in three vials. The amount of particles is quantified as described in Fig. S3. ArfGAP3 is added in two of the samples at different concentrations (50 and 10 nM) corresponding to fractions equal to 0.5 and 0.1 of the Arf1 concentration. Colocalization of coat–Alexa 647 and TAG-Bodipy is lost over time compared with the control.

Hyperreactivity of LDs after COPI action could have several physiological implications. Cytosolic proteins may directly bind to the mother LD surface. Alternatively, LDs might fuse with surrounding organelles, such as other LDs or bilayer membranes. This mechanism may explain the COPI dependence of targeting ATGL-related LD proteins (Fig. S8). For instance, COPI-induced budding of nanodroplets and the resulting decrease in PL packing might lead to the formation of connections between LD and other bilayer membranes, typically endoplasmic reticulum, through which proteins can exchange. These connections have been suggested in various systems by fluorescence recovery after photobleaching and EM experiments (10, 23, 24). Such a mechanism would probably entail a regulation of the action of COPI so that it is active only whenever the targeting of a specific enzyme is required.

Discussion

The fact that COPI machinery is able to work on monolayers and bud oil droplets is a unique and apparently an intrinsic capability of the machinery. Because of the specificity of a monolayer compared with a bilayer, we propose that the COPI machinery performs a previously unrecognized function, clamping the surface tension of the monolayer at a buffer/oil interface by preventing it from dropping down to low tensions, below 2 mM/m. At the molecular level, this action of COPI prevents the LD surface from being fully covered by PLs and provides more accessibility to TAG for binding/reacting with other components. Consistent with the specificity of LD phenotypes associated with depletion of COPI subunits (4, 6), this is likely the only protein that can perform this function: COPIII depends on a transmembrane guanine nucleotide exchange factor, Sec12, whereas ArfGEPs are peripheral proteins that might directly bind to LDs; clathrin coats assemble on membranes rich in anionic lipids, whereas the surface of LDs is very poor in such lipids (18).

Materials and Methods

Proteins. Fluorescently labeled Arf1 was generated by using an Arf1 variant, where the single cysteine residue of Arf1 was replaced with serine, and the C-terminal lysine was replaced with cysteine (Arf1-C159S-K181C) (25). Human Arf1-C159S-K181C and yeast N-myristoyltransferase were coexpressed in Escherichia coli supplied with BSA-loaded myristate. Cell lysates were subjected to 35% (vol/vol) ammonium sulfate, and the precipitate, enriched in myristoylated Arf1, was further purified by DEAE-ion exchange. Eluted fractions of interest were concentrated in spin-column filters with a 10-kDa cutoff (Millipore) and fluorescently labeled using Cy3-maleimide (GE Healthcare) according to the manufacturer’s protocol. To remove excess dye, samples were purified by gel filtration using a Superdex 75 column (GE Healthcare).

Recombinant coatamer protein was expressed and purified as described (26). Sf9 insect cells were infected with baculovirus encoding for heptameric coatamer. Coatomer complexes were isolated from the soluble protein fraction by nickel-affinity purification, concentrated in spin-column filters with a 250-kDa cutoff (Millipore), and fluorescently labeled using AlexaFluor-647-NHS (Molecular Probes) according to the manufacturer’s protocol. Excess imidazole and dye was removed by gel filtration using a Superose 6 column (GE Healthcare).
Buffer.

nanodroplet size measured by EM is 60 nm no longer surrounded by a layer of coat as in 1) for 10 min. Sixty-nanometer TAG nanodroplets can be identified by their size distribution as in 2) that are sometimes shaped as a coat assembly. Large TAG drops (C) are sometimes observed by negative staining EM. Particles were extracted from buffer drops containing unlabeled Arf1 (100 nM), coatomer (COPI, white arrows). Two different fields are shown. (Inset) Magnification of one budded nanodroplet surrounded by a structure shaped as a coat assembly. Large TAG drops (Left) that are sometimes extracted during the process (probably by shear) are not surrounded by a coat. (Scale bar, 100 nm.) (B) The sample recovered under the same conditions as in A was treated with a large amount of ArfGAP3 (ArfGAP3/Arf1 = 1) for 10 min. Sixty-nanometer TAG nanodroplets can be identified but are no longer surrounded by a layer of coat as in A. (Inset) Magnification view of a droplet without coat. (Scale bar, 100 nm.) (C) Size distribution of 278 nanodroplets from 20 EM images similar to that in A. The average lipid nanodroplet size measured by EM is 60 ± 15 nm.


Unless specified, PL/TAG is fixed at 0.5% (wt/vt).

Buffer. Unless otherwise indicated, experiments were performed in HKM buffer: 50 mM Hepes, 120 mM Kacetate, and 1 mM MgCl2 (in Milli-Q water).

Preparation of Synthetic Liposomes and Droplets for Flotation Experiments. For synthetic liposomes, a chloroform solution containing 1 μmol egg PC and 1.6 nmol Rhodamine-PE was dried under argon gas in a glass tube. The lipid film was resuspended in 1 mL HKM buffer. After five cycles of freezing and thawing, the liposome suspension was extruded 19 times through a 0.4-μm polycarbonate filter.

For synthetic droplets, 70 μL TAG was mixed in a glass tube with 0.5 μmol egg PC and 1.6 nmol Rhodamine-PE from stock solutions in chloroform. The solvent was removed using a stream of argon gas and then 0.93 mL of HKM buffer was added to the TAGPL mixture. An emulsion was obtained by vortex and extruded nine times through a 1-μm polycarbonate filter. After extrusion, the emulsion becomes extremely turbid. Examination of the suspension under a microscope shows micrometer-size LDs that were stable for many hours.

Buffer Drops Preparation. Two syringes were filled, one with oil and the other with buffer and proteins. Using a syringe pump, streams from both syringes were allowed to flow into a high-pressure T connector with a 250-μm inside diameter constructed of fluorinated ethylene propylene (27) (Fig. 51).

Because of the wetting properties of the oil to the T connector and the tube, and the ratio of the flow rates (oil rate/buffer rate = 5), 250-μm buffer droplets are generated at the outlet of the T connector (28) and circulate in a transparent Teflon tube where observations were made. The time of reaction action is the time spent by each microreator in the tube; the length of the tube and the flow rate controls it. The flow rate was 1,250 μL/h, and the length of the tube was 2 m. The diameter of the tube was 250 μm, so the reaction time was ~15 min.

GUWs and Micrometer-Scale LDs. GUWs were prepared using an electroformation technique (29). One microliter of PL mixture in chloroform at 0.3 mM was dried on an indium tin oxide (ITO)-coated glass plate. The lipid film was desiccated for 1 h. The chamber was sealed with another ITO-coated glass plate. The lipids were then rehydrated with a sucrose solution (300 mM). The alternative (8 Hz) voltage between the two glass plates was increased by steps every 6 min: 100 mV, 200 mV, 300 mV, 500 mV, 700 mV, 900 mV, and 1.1 V. The last voltage was maintained for at least 1 h. GUWs were either stored in the chamber at 4 °C overnight or directly collected with a Pasteur pipette.

The LDs used in experiments with GUWs (Fig. 4C) were prepared by first drying PLs and solubilizing them afterward into TAG to obtain the required PL/TAG ratio. A mixture of 5 μL of this PL/TAG solution and 95 μL buffer was first vortexed and then sonicated using a Branson 2510 sonicator working at 40 kHz for 20 s. The diameter of the resulting droplets is a few hundred nanometers.

GUWs and generated LDs were incubated together in buffer using 10 μL/min with gentle shaking and subsequently observed under an optical microscope (Fig. 4C is an example of a GUV incubated with LDs at a 0.3% PL/TAG ratio).

LDs and Membranes Mimicking Organelles. To obtain membranes resembling organelles in terms of shape (curvatures) and tension (low tension), 5 μL PL lipid in chloroform (3 mM) was dried on a coverslip and placed in a desiccator for 1 h. The lipid film was rehydrated in 20 μL of buffer for 10 min. The solution was collected and injected into a Petri dish.

To prepare giant LDs, PL mixtures were dried and dissolved into TAG at different concentrations (wt/vt, typically from 0.2 to 5%). Then 5 μL of the oil solution was added to 95 μL of buffer and the mixture was vortexed 10 s using a vortexer (300 rpm) at maximum power.

During the experiment, the LD and the membrane were manipulated and brought into contact through two pipettes via aspiration and observed under a microscope (bright field and fluorescence). Note that LDs were injected last into the Petri dish because they tend to float at the buffer/air interface, where they often spread.

Flotation Assay. Liposomes (120 μL, 1 mM phospholipid) or LDs (120 μL, 0.5 mM phospholipid; TAG/buffer 7/93 vol/vol) were mixed with myristoylated Arf1GDP (0.5 μM) in a final volume of 125 μL. When indicated, the suspension was supplemented with 100 μM GTP and 2 mM EDTA to promote GDP to GTP exchange on Arf1. After incubation for 30 min at room temperature, the sample was adjusted to 30% (wt/vol) sucrose and covered with two cushions of 25% (wt/vol) sucrose (200 μL) and 0% sucrose (50 μL), respectively. The samples were centrifuged for 80 min at 30,000 rpm in a SW60 rotor (Beckman). The top (100 μL) medium (200 μL) and bottom (250 μL) fractions were collected and analyzed by SDS/PAGE using SYPRO Orange staining.

EM. Nano LDs were collected as indicated in Fig. 51. Samples of 5 μL were absorbed to continuous carbon-coated grids (glow discharged) at room temperature for 1 min, rinsed briefly with HKM buffer, and stained with 1% (vol/vol) uranyl acetate for 20 s. Negatively stained samples were imaged under low-dose conditions in a FEI Tecnai12 microscope (120 kV). Micrographs were collected at 26,000× magnification, giving an unbinanced pixel size of 4.2 Å. The diameters of nano LDs were manually measured directly from the micrographs.

Fluorescence Cross-Correlation Spectroscopy. Oil was labeled with 1% vol/vol Bodipy (excited at 488 nm). Coatomer was labeled with Alexa 647 (excited at 632 nm). The product of reaction was recovered as described in Fig. 51 and analyzed with the FCS setup, Confocor2, on the Zeiss microscope LSM510. The emission and excitation spectra of the two dyes are separated enough to not cause any cross-talk or FRET. For each channel, autocorrelation curves were recorded simultaneously within 30 s for many runs of different samples (representing 10 different experiments). Similarly, a cross-correlation curve was simultaneously generated in a third channel. For the cross-correlated signals, we fit the far-red and green autocorrelation curves, G(r), with a theoretical model comprising three components, giving, therefore, three diffusion times (30). The choice of such a number of fitting parameters is
driven by the fact we may have signals from the free dye, the budded particles, and probable aggregates or just large, polluting particles.

\[ G(r) = 1 + \frac{1}{N} \left\{ 1 - x - y \left( \frac{1 - \frac{1}{x+y}}{1 + \frac{1}{25r^2}} \right)^2 + \frac{x}{1 + \frac{1}{z^2}} \left( \frac{1}{1 + \frac{1}{z^2}} \right)^2 + \frac{y}{1 + \frac{1}{z^2}} \left( \frac{1}{1 + \frac{1}{z^2}} \right)^2 \right\} \]

The fraction of each particle is given by \((k_x)^2\) and \(N\) yields the total concentration of particles. The diffusion times are \(\tau_1\), and the access of \(\tau_2\) determines the size of the particles by the Stokes–Einstein law,

\[ \text{radius} = \frac{k_B T}{6 \pi \eta \tau_2} \]

where \(k_B\) stands for the Boltzmann constant, \(T\) the temperature (room temperature), \(\eta\) the viscosity of the buffer (1 cSt), and \(\omega_2\) the width of the focal volume (115 nm at 488 excitation and 155 nm at 647 excitation).

**Measurement of the Interfacial Tension of LDs with Micropipettes.** Phospholipids were mixed at the ratio 70% DOPC: 30% DOPE (mol/mol), dried under vacuum for 1 h, and resuspended in TAG. Ten microliters of this solution was vortexed with 200 µL HKM buffer for 30 s. Then 50 µL of this emulsion was injected into a 1-mL HKM buffer drop deposited on a coverslip and observed with optical microscopy.

The interfacial tension (IT) of the droplets was measured using a micromanipulation technique. The device was made up of a micromanipulator and a pipette holder (Narishige). Pipettes were incubated in a 5% (wt/vol) BSA HKM buffer drop deposited on a coverslip and observed with optical microscopy.

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**Compression Isotherm of PL Monolayer at the TAG/Buffer Interface.** The lipid mixture isotherm was carried out using a Teflon Langmuir trough (Minomicro; KSV) equipped with hydrophilic barriers made of polyoxymethylene (Derlin). The Wilhelmy pressure sensor (KSV) was coupled with a paper plate. Dimensions of the trough were 165 × 51 mm. Room temperature during the experiments was 20.5 ± 0.5 °C. The trough took place on an antivibration table in a closed box containing a water-saturated atmosphere to prevent evaporation. Lipid mixture was spread on HKM buffer before gently depositing droplets of TAG solution in chloroform (1% vol/vol). The amount of deposited TAG (0.12 µL) was at least fivefold higher than the amount of TAG required for the interface to be saturated. The so formed mixed TAG–lipids monolayer, at the interface between the very thin TAG film and HKM subphase, relaxed during 5 h before compression. The compression rate was held constant (0.5Å² min⁻¹ mol⁻¹). The pressure was measured with an accuracy of 0.5 mN/m and the molecular area was controlled with 5% accuracy. The monolayer collapse was observed for 48Å² mol⁻¹ molecular area and 36.6 mN/m pressure values. The pressure owing to pure oil spreading was determined to be 15.2 mN/m. We carried out independent measurements of the surface pressure as a function of lipid mixture spread at the TAG–HKM interface formed in a 50-mm-diameter vessel (glass and Teflon). Even if similar observations have previously been reported in the literature (32), it is worth noting that the results were consistent within the experimental errors with those obtained with the trough regardless of the size of the oil reservoir (from oil monolayer up to macroscopic amounts of oil).

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