Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1773 (2007) 784–793
Biochimica et Biophysica Acta
BBA
www.elsevier.com/locate/bbamcr

Rab-regulated interaction of early endosomes with lipid droplets

Pingsheng Liu [a], René Bartz [a], John K. Zehmer [a], Yun-shu Ying [a], Meifang Zhu [a], Ginette Serrero [b], Richard G.W. Anderson [a,*]

[a] *Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390-9039, USA*
[b] *A&G Pharmaceutical Inc, 9130 Red Branch Rd, Columbia, MD 21045, USA*

Received 29 September 2006; received in revised form 18 January 2007; accepted 5 February 2007
Available online 22 February 2007

Abstract

Recent studies indicate that lipid droplets isolated from a variety of different cells are rich in proteins known to regulate membrane traffic. Among these proteins are multiple Rab GTPases. Rabs are GTP switches that regulate intracellular membrane traffic through an ability to control membrane–membrane docking as well as vesicle motility. Here we present evidence that the multiple Rabs associated with droplets have a function in regulating membrane traffic. Droplet Rabs are removed by Rab GDP-dissociation inhibitor (RabGDI) in a GDP-dependent reaction, and are recruited to Rab-depleted droplets from cytosol in a GTP-dependent reaction. Rabs also control the recruitment of the early endosome (EE) marker EEA1 from cytosol. We use an *in vitro* reconstitution assay to show that transferrin receptor positive EEs bind to the droplet in a GTP/Rab-dependent reaction that appears not to lead to membrane fusion. This docking reaction is insensitive to $ATP_{\gamma s}$ but is blocked by ATP. Finally, we show that when GTP bound active or GDP bound inactive Rab5 is targeted to the droplet, the active form recruits EEA1. We conclude that the Rabs associated with droplets may be capable of regulating the transient interaction of specific membrane systems, probably to transport lipids between membrane compartments.
Published by Elsevier B.V.

Keywords: Membrane traffic; Endosome; Rabs; Adiposomes; Lipid droplets

1. Introduction

Scientists have variously designated sites of lipid accumulation in cells as lipid droplets, lipid bodies, lipid inclusions, oil droplets and oil bodies, which reflects the view that these are simple lipid storage compartments [1,2]. Recent proteomic, lipidomic and genetic evidence suggests, however, that these sites are metabolically active organelles with essential roles in cell signaling, membrane traffic and lipid homeostasis. Nearly all prokaryotic and eukaryotic cells are able to store lipids. Moreover, sites of lipid accumulation are associated with specific membrane systems. In eukaryotic cells, lipid accumulation appears to be associated with endoplasmic reticulum (ER) membranes [3] while in prokaryotic cells special regions of the plasma membrane are involved [2]. Therefore, cells appear to have special compartments that contain the molecules necessary to synthesize and degrade specific classes of lipids as well as manage the traffic of these lipids among membrane systems. For this reason, we have proposed the name adiposome to designate a cellular compartment specialized for packaging and distributing various lipids [4]. The lipid droplet in this nomenclature is a lipid filled adiposome.

The adiposome hypothesis predicts that in eukaryotic cells portions of the ER are specialized for both accumulating lipids and distributing them among various membrane systems. They may also be involved in distributing specific classes of proteins [5]. Although lipid droplets seem an unlikely target for membrane traffic because they lack an intact lipid bilayer, the proteins they contain suggest otherwise. These include: TIP47 [6], a protein involved in mannose-6-phosphate receptor traffic [7]; p22, a calcium binding protein implicated in exocytosis [8]; PKD2, a kinase that regulates Golgi–plasma membrane traffic [9]; the caveolar membrane protein caveolin-1, which may transport newly synthesized cholesterol to caveolae [10] as well as lysosomal cholesterol to the adiposome [11]; and multiple

* Corresponding author. Tel.: +1 214 648 2346; fax: +1 214 648 7577.
E-mail address: richard.anderson@utsouthwestern.edu (R.G.W. Anderson).

0167-4889/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/j.bbamcr.2007.02.004

small GTPases that regulate membrane traffic [4]. While the function of these proteins is unclear, it is possible they are linked to the traffic of essential lipids needed for proper membrane function. For example, cholesterol regulates the traffic of the mannose-6-phosphate receptor between the lysosome and the Golgi apparatus [12] through the activity of NADH sterol dehydrogenase-like protein (NSDHL), which is localized to lipid droplets [6] but can traffic to the Golgi apparatus [13]. Sterols are also critical for the traffic of the yeast tryptophan transporter Tat2 to the plasma membrane [14]. The availability of stored or newly synthesized fatty acids generated by acetyl Co-A carboxylase, another class of lipid droplet-associated enzymes [4], is linked to the acylation of proteins involved in yeast vacuolar traffic [15] as well as the maintenance of the nuclear envelope [16]. Thus, feedback loops appear to exist between the lipids synthesized and stored in droplets and specific membrane systems that carry cargo around in the cell.

An unexpected family of proteins in the adiposome proteome is the Rab GTPases [4]. We detected 9 different Rabs by mass spectrometry and four of these were confirmed by immunoblotting. The presence of these Rabs suggests lipid droplets play a central role in membrane traffic. Indeed, overexpression of Rab11 impairs the movement of endosomal cholesterol to sites of cholesterol esterification [17], which appears to take place in droplets [18]. In addition, microinjected Rab GDP-dissociation inhibitor [19] blocks mobilization of endosomal cholesterol. Finally, Rab18 appears to regulate the interaction of ER with lipid droplets [20] as well as the level of neutral lipid in the cell [21]. Here we present evidence that Rabs regulate the interaction of early endosomes with lipid filled adiposomes.

2. Materials and methods

2.1. Materials

pAb α-caveolin-1, mAb α-EEA1, mAb α-Rab4, mAb α-Ral B and mAb α-Rab5 were from BD Biosciences (San Jose, CA). pAb α-Rab11 and mAb α-transferrin receptor were from Zymed Laboratories Inc. (San Francisco, CA). mAb α-ADRP was from Research Diagnostics Inc. (Flanders, NJ). pAb α-Rab18, pAb α-Rab5, and DNAse I-protease inhibitor cocktail set III were from Calbiochem (La Jolla, CA). pAb α-Myc from Upstate (Charlottesville, VA). Alexa labeled second antibodies were from Molecular Probes (Eugene, OR). Bovine liver Rab GDP-dissociation inhibitor (lot #090K4116), PMSF and all nucleotides were from Sigma (St. Louis, MO). Calf serum, fetal bovine serum and DMEM were from Hyclone (Logon, UT).

2.2. Cell culture

CHO K2 cells were cultured in 150 mm dishes in high glucose DMEM containing 10% calf serum, 40 μg/ml proline, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were either grown until confluent for droplet isolation or to 60% confluent for use in isolating endosomes. HeLa cells were grown overnight in high glucose DMEM with 10% fetal bovine serum and antibiotics were cultured in 12-well plates (seeded at 50,000 cells/well) containing a coverslip and grown over night before transfection.

2.3. Lipid droplet purification

Lipid filled adiposomes (designated droplets) were purified as previously described [4] with several modifications designed to increase the purity. Briefly, 10, 150 mm dishes of cells were collected by scraping in ice-cold PBS, re-suspending in buffer A (25 mM Tricine, pH 7.6, 250 mM sucrose), and homogenizing by N_2 cavitation (450 psi for 15 min on ice). A post-nuclear supernatant fraction was obtained by centrifugation at 1000×*g* and loaded into a SW41 tube. This sample was centrifuged at 274,000×*g* for 1 h at 4 °C. The white band (droplet fraction) at the top of gradient (~0.5 ml) was collected and re-suspended in 6 ml of buffer A in a SW41 tube. The droplet fraction was overlaid with 4 ml buffer B (20 mM HEPES, pH 7.5, 100 mM KCl, 2 mM $MgCl_2$) and centrifuged at 274,000×*g* for 1 h at 4 °C. The droplet fraction from this spin (~0.5 ml) was collected, transferred to an Eppendorf tube and centrifuged at 20,000×*g* for 4 min. The clear buffer underlying the white band was removed, the droplets resuspended in 200 μl of buffer B and centrifuged again. This step was repeated a total of 4 times. The droplet fraction was resuspended in 1 ml of buffer B, vortexed to resuspend the droplets, subjected to a second round of washes and finally centrifuged at 265,000×*g* in a TLA 100.3 tube for 5 min to remove any contaminating membranes as previously described [4].

2.4. Endosome purification

CHO K2 cells at ~60% confluency were washed twice with ice-cold PBS and detached gently from the dish using a cell scraper. Cells from 6, 100 mm dishes were pooled into one 15 ml tube and spun for 5 min at 500×*g* at 4 °C. After removing the supernatant, cells were resuspended in 600 μl buffer C (250 mM sucrose, 3 mM imidazole, pH 7.4 containing 1/1,000 dilution of protease inhibitor cocktail set III) and incubated on ice for 20 min. Cells were then broken using a 22.5 gauge needle. Breaking efficiency was determined by phase contrast microscopy. Unbroken cells and nuclei were removed by spinning for 10 min at 1000×*g* at 4 °C. The pooled post-nuclear supernatant fraction (PNS) from 30, 100 mm dishes was transferred to the bottom of a centrifuge tube (volume: 38 ml) and adjusted to a sucrose concentration of 40.6% using a stock solution of 62% sucrose, 3 mM imidazole, pH 7.4. The sucrose concentration was determined with a refractometer. The solution was then carefully overlaid with 12 ml of 35% sucrose, 3 mM imidazole, pH 7.4, followed by 8 ml of 25% sucrose, 3 mM imidazole, pH 7.4 and filled to the top with buffer C. Gradients were centrifuged at 108,000×*g* in a swinging bucket rotor for 3 h at 4 °C. A visible 25–35% interphase enriched for endosomes (early and recycling endosomes) was collected [22,23]. Fractions were used directly or snap-frozen in liquid nitrogen and stored for up to 2 months at −80 °C.

2.5. Preparation of Rab GDP-dissociation inhibitor

In some experiments we used commercial RabGDI and in others we used recombinant protein purified in the laboratory. For the latter, we used *E. coli* strain BL21 (DE3) expressing a His-tagged, bovine RabGDI in a pRESET plasmid, which was kindly provided by Dr. Oliver Ullrich, Hamburg, Germany. Bacteria were grown under standard conditions and processed to lyse the bacteria by freeze–thawing in buffer D (50 mM Na_2HPO_4, pH 8.0, 0.3 M NaCl, 10 mM 2-mercaptoethanol) containing 1 mg/ml lysozyme and 5 μg/ml of DNase I-protease inhibitor cocktail III. The suspension was incubated on ice for 30 min and then sonicated on ice 15 times for 30 s each at 50 Joule Watt-sec before centrifuging for 1 h at 60,000×*g* at 4 °C to remove the cell debris. To purify the His-tagged protein, 45 ml of the supernatant fraction was mixed with 1.5 ml of Ni-NTA beads (Qiagen, Valencia, CA) that had been pre-equilibrated in buffer D containing 10 mM imidazole. The suspension was rotated on a wheel at 4 °C for 1 h. The beads were spun down at 1000×*g* for 2 min and washed (re-suspend and pellet again) three times with 40 ml of buffer D containing 10 mM imidazole, pH 8.0; three times with 40 ml of buffer D containing 10 mM imidazole, 0.3% Triton X-100, pH 6.0; and two times in 40 ml of buffer D containing 10 mM imidazole, pH 8.0. Beads were loaded onto a 10 ml Poly-Prep chromatography column (Bio-Rad, Hercules, CA), washed with 10 bed volumes buffer D containing 10 mM imidazole, pH 8.0, and finally eluted with 20 ml buffer D containing 200 mM imidazole, pH 8.0. Fractions (1.5 ml) were collected and the fractions with the most protein pooled and dialyzed overnight at 4 °C with 3 times buffer change against buffer E (20 mM HEPES, KOH, pH 7.2, 10 mM DTT) in Pierce Dialysis cassettes (Rockford, IL) that had a molecular weight exclusion < 10,000, and stored at −80 °C. Pooled samples were separated by gel electrophoresis and the purity checked by Coomassie blue staining.