



Rab proteins implicated in lipid storage and mobilization

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Abstract

Abnormal intracellular accumulation or transport of lipids contributes greatly to the pathogenesis of human diseases. In the liver, excess accumulation of triacylglycerol (TG) leads to fatty liver disease encompassing steatosis, steatohepatitis and fibrosis. This places individuals at risk of developing cirrhosis, hepatocellular carcinoma or hepatic decompensation and also contributes to the emergence of insulin resistance and dyslipidemias affecting many other organs. Excessive accumulation of TG in adipose tissue contributes to insulin resistance as well as to the release of cytokines attracting leucocytes leading to a pro-inflammatory state. Pathological accumulation of cholesteryl ester (CE) in macrophages in the arterial wall is the progenitor of atherosclerotic plaques and heart disease. Overconsumption of dietary fat, cholesterol and carbohydrates explains why these diseases are on the increase yet offers few clues for how to prevent or treat individuals. Dietary regimes have proven futile and barring surgery, no realistic alternatives are at hand as effective drugs are few and not without side effects. Overweight and obesity-related diseases are no longer restricted to the developed world and as such, constitute a global problem. Development of new drugs and treatment strategies are a priority yet requires as a first step, elucidation of the molecular pathophysiology underlying each associated disease state. The lipid droplet (LD), an up to now overlooked intracellular organelle, appears at the heart of each pathophysiology linking key regulatory and metabolic processes as well as constituting the site of storage of both TGs and CEs. As the molecular machinery and mechanisms of LDs of each cell type are being elucidated, regulatory proteins used to control various cellular processes are emerging. Of these and the subject of this review, small GTPases belonging to the Rab protein family appear as important molecular switches used in the regulation of the intracellular trafficking and storage of lipids.

Keywords: lipid droplet, rab protein, effector, GTPase, GAP, GEF

INTRODUCTION

Rab proteins are cytosolic small molecule GTPases that are recruited to the cytosolic leaflet of intracellular membranes where they function in various cellular processes. Like other small GTPases, their core function is to act as molecular switches cycling between an “on” and “off” mode through exchange of bound GDP for GTP and subsequent hydrolysis of bound GTP to

GDP. This cycling between an “off” (Rab^{GDP}) and an “on” (Rab^{GTP}) state is accompanied by a conformational change that alters its interaction with effector and accessory molecules. Apart from their role as molecular switches, Rab proteins can also be viewed as macromolecular organizers regulating the assembly of protein complexes.

Rab proteins are distinguished by a post-translational modification, prenylation^[1,2], catalyzed by gera-

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nylgeranyltransferase, or geranylgeranyl transferase II which adds two (usually) geranylgeranyl groups to cysteines present at their carboxy termini. A note of interest is that without these lipid modifications, the Rab protein is effectively inhibited. This can be achieved upon addition of statin^[3,4] that impairs the synthesis of the prenyl groups (a byproduct of cholesterol synthesis). Statin is a class of drugs commonly prescribed to patients with elevated cholesterol levels and cardiovascular disease and acts as an inhibitor of HMG-CoA reductase, a key enzyme in cholesterol synthesis. Pleiotropic beneficial effects independent of the lowering of cholesterol levels observed upon administering statins might be explained through inhibition of Rab proteins and other small GTPases^[5] though equally and perhaps likely, also some of its side effects. For prenylation, Rab proteins are first bound by the Rab escort protein (REP) via a conserved region and then presented to the Rab geranylgeranyltransferase^[2]. Once Rab proteins are prenylated, the lipid anchor(s) ensure that Rabs are no longer soluble and here, the REP plays an important role in binding and hiding the geranylgeranyl groups from the hydrophilic environment of the cytosol and in the delivery of the Rab protein to the relevant cell membrane. As many as 70 Rab proteins are predicted in the human genome, with the majority of unknown function. Most are evolutionarily conserved and exist in diverse species (source: NCBI Gene). As an example, Rab5a, is found in mammals, amphibians (*X. laevis* 97.7% sequence identity), fish (*D. rerio* 98.2%), nematodes (*C. elegans* 88.9%), insects (*D. melanogaster* 83.9%), mold (*N. crassa* 72.2%), plant (*A. thaliana* 72.5%), and yeast (*S. cerevisiae* 64.6%). With such a high degree of sequence identity over a broad range of species, this points to Rab proteins as conserved key regulators of cellular events.

Transition from the “off” (Rab^{GDP}) to the “on” (Rab^{GTP}) state is facilitated by a guanine nucleotide exchange factor (GEF) and coincides with the recruitment to its site of action, e.g. a specific membrane domain enabling the Rab protein to bind and assemble effector molecules for specific functions (e.g. membrane mobility, membrane fusion). The corresponding transition from the “on” to the “off” state is catalyzed by a GTPase activating protein (GAP) enabling hydrolysis of bound GTP to GDP. A GDP dissociation inhibitor (GDI) prevents the release of the GDP and thereby reactivation of the Rab protein^[6]. Putatively, each Rab protein has its own cohort of GEF, GAP, GDI proteins, themselves subject to cellular regulation, yielding versatile and interconnected molecular machineries. Targeting information within each Rab protein selectively recruits it to its preferred membrane domain.

This can be a sub-compartment of an intracellular membrane. As an example, the early endosome recruits at least three Rab proteins: Rab4, Rab5 and Rab11. These Rabs proteins do not colocalize or overlap in their function. Rab4 is recruited to a subdomain destined for rapid recycling and Rab11 to one destined for slow recycling whereas Rab5 is recruited to a subdomain destined for transport/conversion to a late endosome^[7]. In this way, although all three exist on the same membrane, they participate in different processes.

Rab proteins share little sequence identity between themselves. Whereas the *N*-terminal region of Rab proteins responsible for binding GTP and hydrolyzing the GTP is essentially conserved with an active site containing serine and glutamine residues, the remainder of the protein varies greatly^[8]. This variability in sequence enables the Rab proteins to bind different effector molecules and to specify their site of action^[9]. Contained within the Rab protein are targeting motifs as well as interaction domains where, for example, the *C*-terminal residues of Rab7 (residues 174–195) target it to late endosomes^[8]. A primary targeting mechanism for Rab proteins is also through their interaction with their corresponding GEF^[10–12] that binds to specific phospholipids that, in turn, serve as membrane targeting domains. The GEFs often possess phosphatidylinositol (PI) binding domains. PI and its phosphorylated variants act as precursors for second messengers, regulation of cellular processes as well as in the recruitment of proteins to the cytosolic leaflet of phosphobilayers. Furthermore, the synthesis and degradation of these PI species is regulated, and consequently, recruitment of the subclass of proteins that bind to these lipids. There is also evidence to suggest that the curvature of the acceptor membrane plays a role in the selection and recruitment of Rab proteins^[13].

At the site of action, apart from binding GEF, GAP and GDI proteins, each Rab protein regulates assembly of proteins that often act as scaffolds for further recruitment of proteins resulting in large molecular complexes (e.g. motor protein complexes). Rab5 has been shown to interact with at least 20 identified proteins through the use of Rab5-GST fusion proteins in affinity chromatography preloaded with either GTP or GDP and after binding to effector molecules, eluted with GDP or GTP, respectively^[14]: Rabaptin-5^[15], RIN3^[16], Rabex-5 GEF^[17], Rabenosyn-5^[18,19], Rabankyrin-5^[20], EEAI^[21], PI-3 kinases hVPS34 and p85 α -p110 β ^[22,23], PI-4, PI-5 phosphatases^[24], kinesin type motor proteins for microtubule dependent movement^[25], APPL1 and APPL2^[26], syntaxin-13^[27], GGAs^[28], Huntingtin and HAP40^[29]. The nature of the function of Rab proteins (cycling between the “on” and “off” state) may make

for only transient interactions with effectors, which from an experimental perspective, are difficult to trap. An elegant approach to visualize Rab6 in its “on” state (Rab6^{GTP}) based on an expressed phage display-derived antibody fused to enhanced green fluorescent protein showed a preferred location of Rab6^{GTP} to Golgi membranes and transport intermediates^[30]. Combined with affinity-based chromatography, such molecular approaches should prove useful in further elucidating the various processes involving Rab proteins and in the biochemical isolation of involved molecular complexes.

THE LIPID DROPLET AS AN ORGANELLE

Cellular organelles (eg. ER, Golgi, mitochondria, peroxisome, nucleus, TGN, endosome system, lysosome, vacuole, chloroplast) have long since been defined and are relatively well characterized whereas the lipid droplet (LD) only recently gained organelle status. Lipid droplets were originally thought to be static reservoirs of neutral lipids (e.g. TGs and CEs) acting as energy stores for times of need and buffers for potentially toxic lipids (e.g. free fatty acids and cholesterol). Coated by a monolayer of phospholipids that shields neutral lipids of the core from the aqueous cytosol, the LD is unique in its composition. It gained its organelle status after it was shown that LDs possess specific coat and regulatory proteins. Like other organelles, LDs also utilize microtubules for intracellular movement from or to the cell periphery and undergo fission and fusion events. The LD is closely linked to the energy state of the cell (reviewed in^[31–34]) and proteins that are recruited to its surface^[35,36] include Rab proteins and other small GTPases, some with well-defined functions.

The mechanism underlying LD formation is not fully understood. A prevailing hypothesis is that LDs originate from the ER^[34]. Here, neutral lipids are synthesized and inserted between the luminal and cytosolic leaflet of the ER membrane. This leads to a bulging of the cytosolic leaflet followed by a fission event of the cytosolic leaflet releasing the LD into the cytosol. As neutral lipids are free to diffuse laterally, a mechanism must be invoked to enable the LD bulge to grow by preventing neutral lipids from equilibrating throughout the vast ER membrane system. One possibility is that LDs form from micro-domains devoid of transmembrane proteins enabling the two leaflets to separate and that further bulging is promoted by curvature “friendly” phospholipids^[31]. Another possibility is to first bud off a vesicular structure using mechanisms similar to those involved in intracellular

vesicle formation (e.g. COPI, COPII and clathrin-coated vesicles) followed by an enlargement of the intra-leaflet space through TG and CE synthesis/insertion (for review, see^[37]). A role for ER membranes in LD biogenesis is supported by the notion that many ER proteins have been ascribed to the surface of the LD^[34]. An alternative explanation for the presence of ER proteins is that lipid droplets often appear in close juxtaposition to other organelles such as the ER, the mitochondria, peroxisomes and membranes of the endocytic pathway^[35,38–41]. LDs can also be encircled and degraded within autophagosomes, a process termed lipophagy, which results in the mass liberation of fatty acids and cholesterol^[42–45]. Finally, some LD coat proteins are delivered to the LD via COPI and COPII mediated trafficking from the ER/Golgi^[46]. This interdependence between the LD and other organelles underscores its role in central functions of the cell including lipid metabolism, metabolic regulation and signaling^[34]. The LD also appears linked to ER stress and to apoptosis, functional connections implied by proteins found associated to its surface.

The elucidation of the LD proteome is a work in progress. Many high-confidence LD-associated proteins have been identified separating into two partially overlapping populations, those that appear functionally associated and those that appear physically associated. Co-immunoprecipitation, yeast-2-hybrid and affinity purification using known LD-associated proteins as bait have yielded multiple proteins and through the application of liquid chromatography tandem mass spectrometry (LC-MS/MS), several hundred putative LD proteins have been described. LDs can be easily purified from cells and tissue due to their buoyant nature through sucrose gradient centrifugation. Following lipid extraction, associated proteins can then be readily detected. While LC-MS/MS is sensitive and capable of identifying several thousands of proteins in a given sample in a quantitative mode, it does so indiscriminately in that it highlights both real LD proteins and contaminating proteins. Assuming that the LD proteome is composed of both abundant (e.g. PLIN1-5) and less abundant proteins (e.g. Rab18), simply thresholding based on relative abundance is not feasible. Rather, the LD fraction needs to be analyzed in the context of the cell homogenate, cytosol and ideally, other protein-rich and/or associated organelles such as the mitochondria and the ER. Proteins can then be assigned to the LD with higher confidence following hierarchical clustering and heat map-based readout or through protein-correlation profiling using known LD markers as guides^[47–49]. There is an upper estimate of about 500 proteins ascribed to the LD surface of which

50 have been independently verified. These are referenced in^[49-64] and reviewed in^[33,65]. A complementary approach and one also used to independently verify LD candidate proteins is fluorescence imaging of endogenous or expressed proteins. The resulting proteome of LDs, pending verification, is likely to differ between cell types and even within the same cell. LDs from adipose tissue^[52,66], differ greatly from intestinal^[67], intramuscular^[68] or macrophage^[62] LDs, as they serve non-overlapping roles. The classic LD binding proteins, the “PAT” proteins (also referred to as PLIN proteins): perilipin, adipocyte differentiation-related protein (ADRP), TIP47; are not on every type of LD. There is even a suggestion that LDs in the same cell may have different proteomes^[69]. The LD proteome is also adaptable, in that the metabolic state of the cell determines the LD proteome^[51]. Regulatory proteins such as the Rab proteins here play important functions.

LD-ASSOCIATED RAB PROTEINS

There has been at least one proteomics-based report of each of these Rabs being on an isolated LD: Rab1a, 1b, 2a, 2b, 3a, 4a, 4b, 5a, 5b, 5c, 7, 8a, 8b, 10, 11a, 11b, 12, 13, 14, 18, 19b, 21, 22a, 24, 31, 34, 35, 39, 40c, and 41^[49-64]. Following on from the discussion above, this review is limited to those that have been confirmed through independent techniques, such as imaging. These are Rab1, 5, 7, and 18. Rab18 is the only true LD resident Rab protein with extensive evidence for a role in LD function. Rab1, 5, and 7 have also been demonstrated to interact with LDs but with more circumstantial evidence, and will be described thereafter.

Rab 18

Rab18 has been shown to localize to LDs both at the light and ultrastructural level. Expression of a fusion protein between Rab18 and enhanced green fluorescent protein showed typical peri-LD staining typical of surface-associated proteins and confirmed by immunoelectron microscopy showing Rab18 in direct association with the monolayer surface^[70,71]. Rab18 appear localized only to a subset of LDs in both adipocytes and non-adipocyte cell lines^[70]. In cell culture experiments, expression of a dominant active mutant form of Rab18 revealed predominant localization to LDs. In contrast, expression of a dominant negative mutant form of Rab18 showed an exclusive Golgi-like distribution^[70-72]. This form specifically enhanced retrograde Golgi-ER transport^[72] and knockdown of *Rab18* through RNA silencing both disrupts the Golgi apparatus and reduces the normal secretion

pathway^[72]. It has been shown that Rab18 expression increases during differentiation in 3T3-L1 cells^[73] and that insulin treatment induces recruitment of Rab18 to LDs. Overexpression of Rab18 also increases basal lipogenesis, while knockdown of *Rab18* appears to impair the lipogenic response to insulin. This suggests a role for Rab18 in promoting TG accumulation^[73]. Evidence also exists to support a role for Rab18 in lipolysis^[71,74] and that Rab18 levels in adipose tissue correlate with obesity as well as with gender^[75]. Despite being well characterized as a LD-associated protein, the function of Rab18 is yet to be determined. At least three possible roles can be envisaged: 1) A regulatory role in LD-ER interaction; overexpression of Rab18 causes a close apposition of LDs to the rough ER with possible implications in the storage and mobilization of lipid esters in LDs^[71]. 2) A regulatory role for Rab18 in the fusion and fission of LDs; lipogenic and lipolytic stimulation associate with increased motility^[76-79] and increased fusion and fission^[77] events of LDs, respectively. As Rab proteins have been implicated in the regulation of SNARE-dependent fusion events elsewhere in the cell, it is possible that the observed stimulatory role of Rab18 in fusion and fission of LDs involves the regulation and recruitment of SNAREs and associated proteins^[64]. 3) A regulatory role in lipolysis through ATGL; Rab18 is recruited and localized to LDs undergoing lipolysis and evidence suggests that Rab18 recruits ATGL, the primary lipase for LD TG hydrolysis, to the LD in a COPII-dependent manner^[46]. Visualization of *de novo* LD synthesis by fluorescence microscopy^[70] also shows that Rab18 does not interact with LDs at an early stage. Only once the LDs are larger, does Rab18 bind to LDs. These observations suggest that Rab18 is not involved in LD formation (e.g. budding from ER) or initial homotypic fusion events of small LDs.

The role of Rab18 in LD function(s) is highlighted in several diseases (for a complete review of diseases associated with lipid droplet abnormalities, please see^[53]). Hepatitis C virus (HCV) assembly is dependent on the association of core proteins with LDs. Using a proteomics-based strategy, Salloum et al. found that Rab18 interacts with the HCV nonstructural protein NS5A^[80]. The Rab18^{Q67L} dominant active mutant bound more strongly to NS5A than did the Rab18^{S22N} dominant negative mutant. These results support a model in which the specific interactions of the viral protein NS5A with Rab18 promote the physical interaction between assembling HCV virions and lipid droplets^[80]. Hepatitis B virus X protein (HBx) promotes development of hepatocellular carcinoma (HCC) through induction of dysregulation of lipogenesis. In this study,

You et al. showed that the expression levels of Rab18 were positively correlated with HBx expression levels in clinical tissues, and HBx induced expression of Rab18 in *p21-HBx* transgenic mice and hepatoma cell lines^[63]. This study also demonstrated that cyclooxygenase-2 and 5-lipoxygenase were able to stimulate the *Rab18* promoter through activating transcription factor activator protein 1 (AP-1) and cyclic adenosine 3',5'-monophosphate response element-binding protein (CREB). In addition, the authors found that miR-429, a small noncoding microRNA molecule, was able to bind to the 3' untranslated region of Rab18, regulating its expression level. As HBx was shown to down-regulate miR-429 in hepatoma cells, this opens up the possibility that HBx enhances hepatocarcinogenesis through the dysregulation of lipogenesis and proliferation of hepatoma cells, involving two pathways, HBx/COX-2/5-LOX/AP-1/CREB/Rab18 and HBx/miR-429/Rab18^[63].

Additional clues to the role of Rab18 comes from studies of the Warburg micro syndrome, an autosomal-recessive developmental disorder characterized by brain, eye, and endocrine abnormalities^[81]. Previous studies suggested that mutations in *Rab3GAP1/2* were causative for disease development^[82,83]. Bem et al. performed autozygosity mapping in consanguineous families without RAB3GAP1/2 mutations and identified loss-of-function mutations in Rab18 (Leu24Gln, founder mutation; a homozygous exon 2 deletion resulting in a frame shift mutation; an anti-termination mutation of the stop codon X207QextX20; and an in-frame arginine deletion Arg93 del)^[84]. The clinical features of Warburg micro syndrome patients with RAB3GAP1 or RAB3GAP2 mutations and Rab18 mutations are indistinguishable, although no interaction between Rab3 and Rab18 has been demonstrated. It is therefore possible that RAB3GAP1 and RAB3GAP2 act on Rab18 though no evidence exist to suggest that these activating proteins can be found on the LD.

Rab18 may also be indirectly involved in a number of other diseases involving LDs including lipodystrophy, cachexia, neutral lipid storage disease, metabolic syndrome, NASH and atherosclerosis (reviewed in^[53]), but these will have to be independently confirmed. With regards to the known involvement of Rab18 in disease, it is important that the role of Rab18 is elucidated at the mechanistic and molecular level.

Rab 1

Rab1 (with two isoforms Rab1a and Rab1b) has been implicated in multiple intracellular trafficking processes, including transport between ER and

Golgi^[85], autophagosome formation^[86], early endosome motility and sorting^[87], and tethering of p115 to SNAREs^[88]. Rab1a recruitment of the tethering factor p115 into a SNARE complex promotes COPII vesicles budding from the ER for their fusion with Golgi membranes and or recycling Golgi-derived COPI vesicles^[88,89]. It has been shown that Rab1b interacts with the COPII components Sec23, Sec24 and Sec31 and that inhibition of Rab1b changes the COPII phenotype, suggesting a key regulatory role^[90]. It has also been shown that Rab1a is associated with early endocytic vesicles and required for their microtubule-based motility^[87]. In *Rab1a* knockdown cell lines, endocytosed ligands failed to segregate from their receptor and consequently did not reach lysosomes for degradation, indicating a defect in early endosome sorting. Rab1a recruits the minus end directed kinesin motor, KifC1, to early endocytic vesicles^[87]. None of these described functions associates Rab1 with the LD. In contrast, Rab1 and its cognate GAP, TBC1D20, are recruited to LDs and essential for HCV assembly and virulence^[91]. Expression of a dominant negative mutant of Rab1 also inhibits LD formation though whether this is through the interdependence between the biogenesis of LDs and COPI and COPII components is unclear^[91].

Rab 5

Rab5 (with three isoforms Rab5a, Rab5b and Rab5c) has been implicated in multiple intracellular trafficking processes, including early endosome sorting^[18,92], recruitment of SNAREs and tethering^[93,94]. Interestingly, none of these studies suggests an interaction with LDs. Based on the current literature, there is no verifiable physiological mechanism that would include the interaction of early endosomes and LDs, despite many proteomic studies that suggest otherwise. Studies demonstrated that Rabs associated with LDs regulate membrane traffic^[95,96]. Using an *in vitro* reconstitution assay, Liu et al. took advantage of the ability to remove Rabs by the Rab GDI in a GDP-dependent reaction, and to recruit Rabs to Rab-depleted LDs from cytosol in a GTP-dependent reaction^[95,96]. This method can selectively measure the recruitment of Rabs and their effectors to LDs. They showed that both GTP bound active or GDP bound inactive Rab5 are targeted to LDs, but that only the active form recruits EEA1. They proposed that the Rabs associated with LDs may be capable of regulating the transient interaction of specific membrane systems, probably to transport lipids between membrane compartments^[95,96]. Like Rab 18 and Rab1, Rab5 is also implicated in HCV assembly and virulence on LDs^[97].

Rab 7

Rab7 (with three isoforms Rab7a, Rab7b and Rab7c) has been implicated in multiple intracellular trafficking processes, including protein sorting in early endosomes^[98], autophagosome and endosome maturation^[98], as well as receptor and ligand trafficking at the interface/transition between late endosomes and lysosomes^[99,100]. The current view is that Rab5 and Rab7 cooperate in a functional handoff of cargo that is destined for lysosomal degradation^[101]. Here, Rab7 is necessary for recruitment of effectors that promote the maturation of the late endosome^[102,103]. A possible role for Rab7 in the context of LDs comes from the notion that under certain conditions, LDs are targeted and engulfed by autophagosomes and delivered to the lysosomes for lysosomal acid lipase-mediated degradation^[42-45]. This process temporarily puts Rab7 in close apposition to LDs, although perhaps not directly on the LD monolayer surface. Like Rab18, Rab1 and Rab5, Rab7 is also implicated in HCV assembly^[97].

CONCLUSION

Rab18 is the hallmark Rab protein of LDs, while Rab1, Rab5, and Rab7 are candidate LD interacting Rab proteins. Clearly, elucidation of these and other Rab proteins is needed in the context of LD function. Rab mediated recruitment of scaffolding proteins regulates interactions with other organelles, motility and fusion, as is also the case in LDs. It is expected that continued functional studies of LD-associated Rab proteins will address mechanisms to treat the modern disease burden of obesity, insulin resistance and atherosclerosis through elucidation of their molecular pathophysiologies.

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