Expression of Sar1b Enhances Chylomicron Assembly and Key Components of the Coat Protein Complex II System Driving Vesicle Budding

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Objective—SAR1b plays a significant role in the assembly, organization, and function of the coat protein complex II, a critical complex for the transport of proteins from the endoplasmic reticulum to the Golgi. Recently, mutations in SARA2 have been associated with lipid absorption disorders. However, functional studies on Sar1b-mediated lipid synthesis pathways and lipoprotein packaging have not been performed.

Methods and Results—Sar1b was overexpressed in Caco-2/15 cells and resulted in significantly augmented triacylglycerol, cholesteryl ester, and phospholipid esterification and secretion and markedly enhanced chylomicron production. It also stimulated monoaoylglycerol acyltransferase/diacylglycerol acyltransferase activity and enhanced apolipoprotein B-48 protein synthesis, as well as elevated microsomal triglyceride transfer protein activity. Along with the enhanced chylomicrons, microsomes were characterized by abundant Sec12, the guanine exchange factor that promotes the localization of Sar1b in the endoplasmic reticulum. Furthermore, coimmunoprecipitation experiments revealed high levels of the complex components Sec23/Sec24 and p125, the Sec23-interacting protein. Finally, a pronounced interaction of Sec23/Sec24 with sterol regulatory element binding protein (SREBP) cleavage-activating protein and SREBP-1c was noted, thereby permitting the transfer of the transcription factor SREBP-1c to the nucleus for the activation of genes involved in lipid metabolism.

Conclusion—Our data suggest that Sar1b expression may promote intestinal lipid transport with the involvement of the coat protein complex II network and the processing of SREBP-1c.

Key Words: lipoproteins ■ metabolism ■ nutrition ■ Anderson’s disease

Intense efforts have been made over the past 2 decades to better understand the physiological events behind chylomicron (CM) formation.1–4 CMs are essential for the transport of alimentary lipids and fat-soluble vitamins to the bloodstream via the lymphatic system. Although there has been significant progress in intestinal fat transport, major issues remain unsolved regarding the sites, steps, and identity of critical protein participants and regulation involved in lipid translocation from the enterocyte to blood circulation. Uncovering this essential information would serve to better ascertain and appropriately treat lipid transport abnormalities, including fat malabsorption and lipoprotein overproduction, which results in dyslipidemia and atherosclerosis.5

Several groups, including ours, have contributed widely to dissecting the multistep CM assembly within the enterocyte.1–4 6 This complex process includes cellular lipid esterification, translocation of cellular lipid pools, biogenesis and posttranslational modification of various apolipoproteins (apo), and, finally, packaging lipid and apo components into CM. Until now, it has been thought that the key process in CM assembly/secretion is the intracellular association of the essential apo B-48 with lipids7 with the obligatory involvement of microsomal triglyceride transfer protein (MTP).8 Mutations of apo B-48 and MTP result in hypobetalipoproteinemia and abetalipoproteinemia, respectively, with impaired lipid transfer from intestinal enterocytes into the plasma, steatorrhea, failure to thrive during infancy, and other symptoms due to deficiencies of essential fatty acids (FA) and fat-soluble vitamins. Our recent studies provided compelling evidence that Sar1b GTPase (commonly named Sar1b) also plays a pivotal role in CM exocytosis from the enterocytes because its mutations lead to CM retention disease (CRD), or Anderson disease,9,10 which shares common clinical characteristics with hypobetalipoproteinemia

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2692
and abetalipoproteinemia. In fact, Sar1b protein rallies coat protein complex II (COPII) to form a shell around the vesicles transporting CM cargo in the secretory pathway for their budding and fusion with the Golgi.

Newly synthesized and properly folded proteins are transported from the endoplasmic reticulum (ER) to the trans-Golgi network via COPII vesicles to reach their final destination.\(^{11-13}\) COPII coats are assembled from 3 components, ie, Sar1b, Sec23/24, and Sec13/31 complexes, necessary to capture cargo proteins and sculpt the ER membrane into vesicles.\(^{14}\) Current models for protein sorting propose that Sar1b is first activated to function as a molecular switch for the recruitment of the 2 additional cytosolic complexes that direct vesicle budding from the ER.\(^{15}\) Sar1b cycles between inactive GDP- and active GTP-bound forms play a key role in the regulation of the formation of COPII-coated vesicles.\(^{12}\)

COPII-coated vesicle biogenesis commences when Sar1b is converted from the GDP- to GTP-bound state, triggering a sequence of recruitment events that assemble the COPII coat and subsequently initiate the budding of COPII-coated vesicles.\(^{16}\)

Sar1b is likely to have a multiple functions in cargo sorting, regulation of COPII coat dynamics, and membrane deformation through the process of vesicle formation.\(^{17,18}\) Although defects in Sar1b were found to be associated with severe lipid absorption disorder in humans, this important protein has not been extensively investigated in relation to intestinal lipid transport and regulation. In particular, no studies have so far performed functional investigations to show that Sar1b may be at the center of crucial pathways of intestinal lipid transport and function. In the present work, functional studies were carried out to determine the regulatory influence of Sar1b on lipid synthesis, apo biogenesis, and lipoprotein formation, as well as on components that contribute to the efficiency of COPII-mediated protein export from the ER.

**Methods**

Experiments were performed using the Caco-2/15 cell line to overexpress Sar1b/GTPase (Sar1b\(^{+/+}\)). The intestinal cells were transduced with lentivirus-Sar1b-green fluorescent protein. After lentivirus infection, cells were allowed to proliferate until reaching confluence and differentiate for 14 days before being tested for Sar1b\(^{+/+}\). Control mock cells were obtained by infection with GFP lentivirus. Reverse transcription–polymerase chain reaction (A) and Western blot (B) assays were carried out, and the quantification of band intensities showed a significant increase in Sar1b gene and protein expression, respectively, in cells that were transduced with lentivirus-Sar1b-GFP compared with Caco-2/15 transduced with GFP only (mock cells). Results represent the means±SEM for n=3 independent experiments and are represented as ratio of Sar1b\(^{+/+}\)/GAPDH or Sar1b\(^{+/+}\)/\(\beta\)-actin. *P<0.001 vs control mock cells.

**Results**

**Sar1b Overexpression and Cell Integrity**

To examine the role played by Sar1b in intestinal lipid metabolism, studies were carried out using lentivirus as an experimental approach to generate a cellular model overexpressing Sar1b. Sar1b expression was measured following clone selection after 14-day differentiation. The infected Caco-2/15 cells exhibited a marked SAR1B gene overexpression and a significant protein increase compared with mock cells infected with green fluorescent protein lentivirus (Figure 1). Because mock cells were comparable to noninfected Caco-2/15 cells in terms of gene and protein expressions (data not shown), only mock cells were used as controls in subsequent experiments.

We then examined how genetic manipulation of Sar1b level altered Caco-2/15 cell integrity. No pronounced alterations were noted in cell viability (assessed with the Trypan Blue dye exclusion method) or transepithelial resistance between mock and Caco-2/15 cells overexpressing Sar1b (data not shown). Similarly, estimation of the expression of the 2 characteristic brush border membrane markers, villin (Supplemental Figure 1A) and sucrase (Supplemental Figure 1B) proteins, did not reveal significant changes. Collectively, these data suggest that genetically modified Caco-2/15 cells are viable, fully differentiated, and endowed with normal permeability and function.

**Measurement of Lipid Esterification**

To elucidate the impact of Sar1b\(^{+/+}\) on lipid esterification and delivery, Caco-2/15 cells were cultured for 24 hours with \(^{14}\text{C}\)-oleic acid. As illustrated in Supplemental Figure II, substantial changes were recorded in the major lipid classes in cells and media. The augmentation in Sar1b expression led to a significant augmentation in cellular triacylglycerols (TGs) (Figure 2), cholesteryl ester, and phospholipids (Sup-
papillalipid (Supplemental Figure II). Together, these findings suggest a role of Sar1b+/- in lipid synthesis and secretion.

Apo Biosynthesis and Secretion

The subsequent step was to examine the modulation of apo production in intestinal epithelial cells overexpressing Sar1b. To this end, Caco-2/15 cells were cultured with [35S]methionine for 24 hours, and the delivery of newly synthesized apoprotein in the medium was analyzed by SDS-PAGE following immunoprecipitation. On the incubation of Caco-2/15 cells with the radiolabeled precursors, a rise in the synthesis and secretion of apo B-48 (Figure 2) and apo B-100 was observed (Supplemental Figure IIIA). A similar trend characterized apo A-IV (Supplemental Figure IIIB) without significant changes in the production and output of [35S]-methionine-labeled apo E (Supplemental Figure IIIC).

Importantly, the ratio of apo B-48 (the main apo B form)/TGs in the medium revealed a significantly (P<0.001) diminished value of Sar1b+/- cells (2.50±0.32) compared with mock cells (4.10±0.37), suggesting an increased lipoprotein size of TG-rich lipoprotein particles.

MTP

Because the assembly of TG-rich lipoproteins represents a complex process in which apo B-48 is packaged with lipids with the obligatory involvement of MTP, we examined the activity of this key protein to gain insight into the mechanisms responsible for the induced apo B-containing lipoprotein assembly and secretion. Both the protein expression (Figure 2) and the activity (Figure 2) of MTP were found to be elevated in Caco-2/15 cells overexpressing Sar1b.

Lipoprotein Assembly

To determine whether Sar1b+/- was able to regulate lipid transport, Caco-2/15 cells were incubated with [14C]oleic acid for 24 hours, medium was collected, and lipoprotein fractions were immediately separated by ultracentrifugation. Figure 3 shows that Sar1b+/- in Caco-2/15 cells resulted in a consistent delivery increase in the CM (2-fold, P<0.01) and very-low-density lipoprotein (1.8-fold, P<0.01) fractions.

Monoacylglycerol Acyltransferase and Diacylglycerol Aciyltransferase Activity Assay

The rise in intracellular production of TGs prompted us to determine whether the activity of monoacylglycerol acyltransferase and diacylglycerol acyltransferase, involved in FA esterification, was influenced by Sar1b forcing. Caco-2/15 cells with Sar1b+/- displayed a significant increase in the activity levels of monoacylglycerol acyltransferase (Supplemental Figure IVA) and diacylglycerol acyltransferase (Supplemental Figure IVB) enzymes compared with controls.

Process Specificity

To test whether all the lipid and lipoprotein processes observed in response to Sar1b overexpression were specific, we have evaluated the impact of Sar1b+/- on cellular incorporation of various nutrients. No significant changes were observed between mock and genetically modified cells in the uptake of calcium (2.22±0.17 versus 2.34±0.20...
pmol/mg protein per minute), glutamine (209.45 ± 17.22 versus 188.33 ± 21.23 pmol/mg protein per minute), and deoxy-
glucose (563.3 ± 37.3 versus 548.1 ± 42.5 pmol/mg protein
per minute), respectively. Therefore, one cannot argue that
the overexpression of this gene globally generates higher cell
functions.

**Does Sar1b Overexpression Have an Impact on
Sar1a Protein Mass?**

In mammals, there are 2 isoforms of Sar1, Sar1a and Sar1b. The
second human protein isoform of Sar1, Sar1a, is encoded by the
SAR1A gene (OMIM 607691). To determine whether Sar1b
forcing influences the expression of Sar1a, we assessed the mass
protein of the latter. Western blot analysis could not reveal any
changes in Sar1a protein expression in response to Sar1b
overexpression in Caco-2/15 cells (Supplemental Figure V).
These findings clearly exclude the interference of Sar1a with the
TG-rich lipoprotein assembly mediated by Sar1b.

**Sar1b Forcing and Involvement of
COPII Components**

As mentioned before, the COPII coat consists of the small
GTPase Sar1b, the Sec23/24, and the Sec13/31 complexes
that sequentially bind to the ER membrane. We were first
interested to determine whether Sar1b overexpression alters
the expression of Sec12 because this ER-localized transmem-
brane protein catalyzes GDP/GTP exchange on Sar1b, result-
ing in GTP loaded Sar1b that recruits the cargo adaptor
complex Sec23/Sec24 to ER exit domains. Using Western
blot, a substantial increase was noted in the protein mass of
Sec12 in the microsomal fraction prepared following cellular
Sar1b forcing (Figure 4A). We thereafter turned to Sec23,
which represents the GTPase-activating protein that stimu-
lates the enzymatic activity of Sar1b. Once again, analysis
by Western blotting revealed an abundant quantity not only of
Sec23 (Figure 4C) but also of Sec24 (Figure 4D) in micro-
somal fractions, but without any significant changes in Sec13
(Figure 4B) and Sec31 (Figure 4E) protein expression. We
finally focused on p125A protein, which is required for
Sec23 interaction, and detected a raised protein level in
the microsomal fraction of cells with Sar1b overexpression
(Figure 4F).

To determine the impact of Sar1b overexpression on the
interaction of various components that trigger the COPII
machinery, we used the coimmunoprecipitation technique.
This experimental approach allowed us to show a more
intense cooperation between the Sec12 guanine nucleotide

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**Figure 4. Impact of Sar1b overexpression on microsomal protein mass of coat protein complex II components, the guanine nucleotide
exchange factor Sec12, and the Sec23-interacting protein p125A in Caco-2/15 cells. Microsomal fractions were prepared from control
mock and Caco-2/15 cells overexpressing Sar1b. The microsomal proteins (50 μg) were fractionated by SDS-PAGE and electrotransferred
onto Hybond-ECL nitrocellulose membranes that were blocked in Tris-buffered saline (pH 7.5) containing 0.1% Tween 20 and
5% nonfat dry milk. The blot was then incubated overnight at 4°C in a blocking solution with the specific antibodies. Immune
complexes were revealed by means of horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin (IgG) and an enhanced chemi-
luminescence kit. Protein mass was quantitated by using an HP Scanjet scanner equipped with a transparency adapter and software.
Values are expressed as mean ± SEM for n = 3 separate experiments. * P < 0.05, ** P < 0.02 vs control mock cells.
Finally, for validation purposes, we isolated the pre-CM transport vesicles from the ER of Sar1b+/+ cells, as reported previously.22–26 The characterization of these vesicles revealed various proteins that collocate with apo B-48, including liver-FAd-binding proteins and CD36, as well as Sar1, Sec12, Sec23/24, and p125A (Supplemental Table I).

Discussion

Intensive work on genetic disorders of fat transport has afforded new insights into the mechanisms involved in intestinal lipid handling and lipoprotein metabolism. Three inherited disorders, namely abetalipoproteinemia, hypobetalipoproteinemia, and CRD, have provided significant advances in our understanding of human physiology related to the assembly of TG-rich lipoproteins. Although their molecular genetic causes have been defined and it is particularly stressed that MTP, apo B, and Sar1b are essential and key components of the TG-rich lipoprotein assembly pathway, relatively few functional studies at the molecular and cellular level have been reported specifically on CRD. To tackle this issue in the present work, we have successfully overexpressed Sar1b in Caco-2/15 cells and can for the first time document that the stable forcing of Sar1b is tightly associated with the amplification of lipid transport. Mechanistically, we were able to highlight the Sar1b-mediated induction of apo B and MTP to augment the assembly of TG-rich lipoproteins. Our findings also emphasize the requirement of particular proteins, such as Sec12, p125A, and Sec23/Sec24, to accommodate Sar1b overexpression for the stimulation of lipid transport via lipoprotein assembly and secretion. Finally, the lateral movement of SREBP-1c into COPII-coated vesicles on ER membranes seems to be implicated in the regulation of lipid processing.

The secretion process of lipoproteins has been largely described in Caco-2 cells under different physiological conditions. Studies from various laboratories have reported the low capacity of Caco-2 cells to transport lipids under the form of lipoproteins. About 15% to 20% of TGs are exported, whereas residual TGs are retained, as was the case in the present investigation. On the other hand, Figure 2 documents the total FA incorporation and esterification, which indicates higher FA uptake in Sar1b+/+ cells than in control mock cells.

The formation of CMs, the largest lipoproteins synthesized exclusively by intestinal epithelial cells, is crucial for the transport of alimentary lipids and fat-soluble vitamins. To date, mechanisms by which lipids are recruited during CM assembly are incompletely understood. According to the prevailing concept, association of apo B-48 with lipids is mediated by MTP in the rough ER, resulting in a lipid-poor, small apo B-48-containing particle that serves as a precursor of mature CM. Our laboratory has not only contributed to illustrating the essential role of apo B-48 and MTP but also been able to point out the absolute requirement of Sar1b for CM assembly.9,10,27 Mutations of the SARA2 gene coding for Sar1b lead to CRD, characterized by varying degrees of chronic fat malabsorption, hypcholesterolemia, fat-soluble vitamin deficiency, failure to thrive, chronic diarrhea, and neurological manifestations.6,10,27–30 Importantly, the intracel-

Figure 6. Coimmunoprecipitation of Sec23, Sec24, SCAP, and sterol regulatory element binding protein-1c (SREBP-1c) in Caco-2/15 cells. To determine the direct interactions of Sar1b with important proteins required for coat protein complex II formation, the coimmunoprecipitation technique was used. Microsomal proteins (50 μg) were reacted with Sar1b antibodies. The immunoprecipitates were subjected to gel electrophoresis and transferred to nitrocellulose membranes. Thereafter, the latter were blotted with antibodies directed against Sec23, Sec24, SCAP, and SREBP-1c.
ular traffic of apo B-48-containing CM is dependent on the COPII coat complex, which buds vesicles from the ER membrane to transport newly synthesized proteins to the Golgi apparatus. Because COPII vesicle biogenesis and assembly begins when Sar1b triggers a sequence of recruitment events that assemble the COPII coat from its cytosolic components and ultimately drives vesicle budding,12 we have reasoned that Sar1b27-29 may influence intestinal lipoprotein trafficking and sorting. Indeed, the present experiments reveal a stimulation of CM assembly and output in the presence of abundant Sar1b in Caco-2/15 cell line. Therefore, it seems that by being critical for COPII functioning, Sar1b GTPase action represents a limiting step that is determinant for the routing/movement of apo B-48-containing CM from the ER to the Golgi apparatus. Sar1b likely activates cargo sorting and completion of the final fission plays to fuel apo B-48-containing CM trafficking.

Apo B-48 is the principal protein component of CM and is cotranslationally lipitated by MTP.31 The lipidation process may be facilitated by the addition of TGs, and when this occurs, the production of apo B-48 and CM increases. Accordingly, it was shown that enhanced apo B output likely reflects adequate lipidation of apo B by MTP, a critical player in the early posttranslational regulation of apo B.32 One can therefore assume that proficient Sar1b efficiently drives COPII machinery action capable of diverting apo B-48 to the secretory pathway and promoting CM processing and secretion. Additional studies are needed to delineate the mechanisms of action of Sar1b, because the information on the processes of translational apo B-48 in the intestine is not as abundant as the apo B-100 in the liver, which is retrotranslocated to the cytoplasm through the translocon in lipid-poor situations, ubiquitylated, and degraded by the proteasome.33 Nevertheless, the available literature allows us to mention that apo B-48 may be regulated by the ubiquitin-proteasome pathway and cytosolic hsp70. Our previous observations suggest that the development of insulin resistance and diabetes stimulated the intestinal transport of lipids by augmenting apo B-48 synthesis through reduced proteasomal degradation activity, resulting in enhanced TG-rich lipoprotein assembly and secretion.5 Moreover, Fischer’s group showed that hsp90 facilitates the interaction between ER-associated apo B and components of the proteasomal pathway, perhaps in cooperation with hsp70.34 In the present work, the presence of the proteasome inhibitor benzyloxycarbonyl-Leu-Leu-leucinal (MG132) at the concentration of 50 μmol/L was ineffective in augmenting the levels of apo B-48 (data not shown), suggesting a direct effect of increased biogenesis rather than any involvement in proteasomal degradation activity in response to Sar1b overexpression.

ER export is mediated by vesicle formation at specialized ER domains known as the ER exit sites by the COPII complex. COPII subunits must first be recruited to the correct sites on the ER membrane, ie, that of the ER exit sites. The COPII coat is formed through sequential binding of 3 cytosolic components, a small GTPase Sar1,15,19-21 the Sec23/Sec24 heterodimer complex,25 and the Sec13/Sec31 heterotrimeric complex,26 to the ER exit sites. Following Sar1-GTP binding to the Sec23/24p complex via Sec23p, Sec24p captures the cytoplasmically exposed signal of the transmembrane cargo27,28 to form a prebudding complex.29 Sec23p is the GTPase-activating protein for Sar1 and therefore stimulates GTP hydrolysis on binding to Sar1,30 leading to disassembly of the prebudding complex.50 Our own findings reinforce the need for increased amounts of Sec23/Sec24 in parallel with the appeal of profuse Sar1b to cope with the transfer of proteins and lipoproteins from ER to Golgi.

Although 2 isoforms characterize Sar1, only Sar1b has been associated with the rare recessive disorder CRD,9 characterized by the selective retention of CM-like particles within the ER of enterocytes.30,41 To date, 11 separate mutations in the SAR1b gene (SARA2) have been detected.42 In some patients’ duodenal biopsies, the fall in Sar1b gene expression was related to Sar1 protein mRNA elevation, an indication of a certain degree of compensation.43 However, in the present investigation, we could not detect any changes in Sar1 protein expression in response to Sar1b overexpression in Caco-2/15 cells.

The sequence of COPII protein assembly was established by the sequential addition of COPII components to an in vitro ER vesicle budding assay.44-46 Even though COPII vesicle formation could be minimally reconstituted using purified COPII proteins,14 additional regulatory factors, such as Sec12 and p125A, are obviously necessary to efficiently support ER-Golgi transport. Indeed, assembly of the COPII coat is initiated through the activation of Sar1 through conversion of its GDP to GTP under the control of Sec12, a guanine-nucleotide-exchange factor for Sar1.46 This GDP-to-GTP transition triggers the exposure of the N-terminal amphipathic α-helix element of Sar1 that inserts into the ER membrane.47-48 On the other hand, p125A was identified as an interacting partner for Sec23A to facilitate ER export from the ER exit sites.49,50 p125 contains an N-terminal proline-rich region responsible for the interaction with Sec23p.51 Our results here show that p125A is a Sec23A-interacting protein and likely part of a Sec23/Sec24/p125A heterohexameric complex, for which additional studies are required to define its actual contribution to the efficiency of COPII-mediated protein and especially lipoprotein export from the ER.

SREBPs are transcription factors that belong to the basic helix-loop-helix leucine zipper family and are considered to be profoundly involved in the transcriptional regulation of cholesterogenic and lipogenic enzymes.52,53 Unlike other members of the basic helix-loop-helix leucine zipper family, SREBPs are synthesized as precursors bound to the ER. To activate lipid synthesis, the SREBPs must be transported to the Golgi, where proteases release the active domains that enter the nucleus. ER-to-Golgi transport requires that the SREBPs form complexes with SCAP, an escort protein.54 On inhibition, SCAP is tightly associated with Insig-1, causing retention of the SCAP/SREBP-1c complex in the ER and thus preventing proteolytic processing of the nascent SREBP-1c. Conversely, on activation, Insig-1 dissociates from the SCAP/SREBP-1c complex, thereby facilitating its transport to the Golgi, where full-length SREBP-1c undergoes proteolysis to generate nSREBP-1c. It has been reported that the SCAP/SREBP complex clusters laterally with other proteins into COPII-coated vesicles that bud from the ER.55
Our studies showed that clustering of the SCAP/SREBP-1c complex is augmented with the overexpression of Sar1b that recruits the heterodimeric protein Sec23/24. Accelerated movement of SREBP-1c would permit connection with genes in the nucleus with the induction of lipid and lipoprotein processing.

Previous studies reported that pre-CM transport vesicles are large COPII-containing vesicles that transport the developing CM from the ER to the Golgi.24,25 Once detached from the ER membrane, the pre-CM transport vesicles dock and then fuse with the Golgi. In fact, the exit step of the pre-CM from the ER is the rate-limiting step in which dietary TG traverses the intestinal absorptive cell.56,57 According to our data, it is reasonable to assume that the presence in abundance of Sar1b, the initiator of the COPII complex, facilitates the formation of the vesicles, their fusion with the Golgi, and lipoprotein trafficking.

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Disclosures

None.

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SUPPLEMENT MATERIAL

Cell culture

The human epithelial colorectal adenocarcinoma Caco-2/15 cell line was obtained from Dr. JF Beaulieu (Department of Cellular Biology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Quebec, Canada). Briefly, Caco-2/15 cells (American Type Culture Collection, Rockville, MD) were grown at 37°C with 5% CO2 in Dulbecco's Modified Eagle's Medium (WISENT BIOCENTER, CA) containing 1% penicillin-streptomycin and 1% Modified Eagle's Medium nonessential amino acids (WISENT BIOCENTER, CA) 1% sodium pyruvate (SIGMA ALDRICH INC.), 1% glutamax (GIBCO BRL), and supplemented with 10% decomplemented fetal bovine serum (Flow, McLean, VA). For experiments, cells were plated at a density of 1x10⁶ cells/well on six-well polycarbonate Transwell filter inserts plates (Costar, Cambridge, MA), permitting separate access to the upper and lower compartments of the monolayer. Cells were cultured for 14 days to fully differentiate for optimal lipid synthesis.1,2 The medium was refreshed every 48 h.

Establishment of Sar1b/GTPase and stable GFP overexpression in Caco-2/15 cell line

The blunt-end PCR fragment encoding Sar1b/GTPase gene was amplified from the Human cDNA clone SC114457 (Origene Technologies, Inc., Rockville, MD) with ATGTCCTTCATATTTG as forward primer and CAGATCCTCTTCTGAGATGAGTTTTTGTTCATCAATGTTACTGTGCCAT containing myc epitope as reverse primer and TOPO Cloned into pLenti6/V5-D-TOPO (Invitrogen Corp., Carlsbad, CA 92008) to create an expression construct. The pLenti6/V5-D-TOPO expression plasmid of Sar1b/GTPase and the ViraPower Packaging
Mix were then cotransfected, using the well known calcium phosphate method, into the 293FT cell line to produce lentivirus. The produced lentiviral stock was used to transduce the mammalian Caco-2/15 cell line. RT-PCR and Western blot assays for transient expression of the recombinant protein were carried on and generation of stable cell line were done using Blasticidin selection at a concentration of 1 μg/ml. Control Mock cells were obtained by infection with GFP lentivirus (kindly provided by Dr Jean-François Beaulieu, University of Sherbrooke) harbouring the same features as the pLenti6/V5-D-TOPO system.

**Immunoblotting analysis**

To assess the presence of various proteins and evaluate their mass, Caco-2/15 cells were homogenized and adequately prepared for Western blotting as described previously. Proteins were denatured in sample buffer containing SDS and β-mercaptoethanol, separated on a 4–20% gradient SDS-PAGE, and electroblotted onto nitrocellulose membranes. Nonspecific binding sites of the membranes were blocked with defatted milk proteins followed by the addition of primary antibodies directed against the targeted proteins. The relative amount of primary antibody was detected with species-specific horseradish peroxidase-conjugated secondary antibody. Even if identical protein amounts of tissue homogenates were applied, the β-actin protein was used to confirm equal loading on SDS-PAGE. Blots were developed and the mass of the various proteins was quantitated using an HP Scanjet scanner equipped with a transparency adapter and software.

The following dilutions of antibodies were used: mouse anti-myc (Santa Cruz, Biotechnology, Inc.), 1/1000; mouse anti-human villin (BD Transduction Laboratories),
Levy: Role of Sar1b in lipid and lipoprotein production

1/2000; mouse anti-β-actin (SIGMA ALDRICH), 1/10000; mouse anti-human sucrase (kindly provided by Dr J-F Beaulieu, University of Sherbrooke), 1/20; anti-mouse IgG-POD/anti-rabbit IgG-POD (Roche Diagnostics, Indianapolis), 1/10000; rabbit anti-Sec12 (Novus Biologicals, Littleton, CO), 1/1000; rabbit anti-p125A (Novus Biologicals, Littleton, CO), 1/3000; rabbit anti Sec13 (Abcam, San Francisco, CA), 1/5000; chicken anti Sec23 (Abcam), 1/500; rabbit anti-Sec24 (Abcam), 1/2000; rabbit anti-Sec31 (Abcam), 1/500; rabbit anti-SCAP (Abcam), 1/500; rabbit ant-SREBP-1c (Abcam), 1/1000.

**Quantitative Real-Time (RT)-PCR**

Experiments for mRNA quantification as well as for GAPDH (as a control gene) were performed using the GeneAmp PCR System 9700 (Life Technologies). Approximately 30–40 cycles of amplification were used at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Amplicons were visualized on standard ethidium bromide-stained agarose gels. Under these experimental conditions relative to RT-PCR, 32–35 cycles corresponded to the linear portion of the exponential phase.

Quantitative RT-PCRs were performed using Quantitect SYBR Green kit (Applied Biosystems, Foster City, CA) in an ABI Prism® 7000 Sequence Detection System. The cycling was as follows: 95°C 10 min, for 1 cycle; 950C 15 s, 60°C 30 s, 72°C 30 s for 40 cycles and 72°C 10 min for 1 cycle. Data was analysed the $2^{-\Delta\Delta CT}$ method. The primers used for either RT-PCR or Quantitative PCR are: Sar1b/GTPase (5’-AGGTTGCGAGAGATGTTTGG-3’ and 5’- ATCAATGTACTGTCCAT-3’); GAPDH (5’- AGAAGGCTGGGGCTCATT-3’ and 5’- GGGCCATCCACAGTCTTCT-3’).

**Measurement of lipid synthesis and secretion**
Levy: Role of Sar1b in lipid and lipoprotein production

Lipid synthesis and secretion were assayed as previously described.\textsuperscript{2, 5, 6} Briefly, radiolabeled \(^{14}\text{C}\)-oleic acid (sp act, 53 mCi/mmol; Amersham, Oakville, ON, Canada) was added to unlabeled oleic acid and then solubilized in fatty acid-free bovine serum albumin [BSA/oleic acid, 1:5 (mol:mol)] containing 0.3 mM monoolein, 4.8 mM taurocholate and 0.5 mM lyso-phosphatidylcholin. The final oleic acid concentration was 0.7 mM (0.45 \(\mu\text{Ci})/well. Cells were first washed with phosphate-buffered saline (PBS) (GIBCO), and the \(^{14}\text{C}\)-oleic acid-containing medium was added to the upper compartment. At the end of a 24-h incubation period, cells were washed, and then scraped with a rubber policeman in a PBS solution containing antiproteases (phenylmethylsulfonyl fluoride, pepstatin, EDTA, aminocaproic acid, chloramphenicol, leupeptin, glutathione, benzamidine, dithiothreitol, sodium azide, and Trasylol, all at a final concentration of 1 mM). An aliquot was taken for lipid extraction by standard methods\textsuperscript{2, 5, 6} in the presence of unlabeled carrier [phospholipids, triglyceride and cholesteryl ester].

The various lipid classes synthesized from \(^{14}\text{C}\)-oleic acid were then separated by thin-layer chromatography (TLC) using the solvent mixture of hexane, ether, and acetic acid (80:20:3, vol:vol:vol), as previously described.\textsuperscript{2, 5, 6} The area corresponding to each lipid was scratched off the TLC plates, and the silica powder was placed in a scintillation vial with Ready Safe counting fluid (Beckman, Fullerton, CA). Radioactivity was then measured by scintillation counting (LS 5000 TD, Beckman). Cell protein was quantified by the Bradford method and results were expressed as disintegrations per dpm per milligram of cell protein. Lipid secreted in the basolateral compartment was analyzed and
Levy: Role of Sar1b in lipid and lipoprotein production

quantified, as described above, after centrifugation (2,000 rpm for 30 min at 4°C) to remove cell debris.

Cholesterol biogenesis was evaluated employing [14C]-acetate as precursor (53.9 Ci/mmol) after a 24-h incubation period. Separation of the cholesterol fraction was performed by TLC.

**De novo apolipoprotein (apo) synthesis**

The effect of Sar1b/GTPase protein overexpression (Sar1b+/+) protein on newly synthesized and secreted apos (A-I, A-IV, B-48, B-100, and E) was assessed as described previously. To first induce apo synthesis, cells were incubated apically with unlabeled oleic acid bound to albumin in serum-free medium, 24 h before [35S]-methionine incubation. The concentration of the unlabeled lipid was equivalent to the labeled substrate described above. After 24-h incubation, cells as well as the outer chambers were rinsed twice with PBS (GIBCO). The apical compartment was replaced with 1.5 ml of methionine-free medium containing the unlabeled substrate and 100 μCi/ml [35S]-methionine (Amersham Life Sciences, 50 mCi/mmol). After incubation for 24 h at 37°C with 5% CO2, the medium from the basolateral compartment was collected. Cells were scraped off the inserts in the cell lysis buffer, as described above. The medium and cell lysates were supplemented with the antiprotease cocktail. To assay a considerable amount of de novo apo synthesis, the material from two wells was pooled.

**Immunoprecipitation of apolipoproteins**

The basolateral medium and cell lysates were first supplemented with unlabeled methionine to act as a carrier (final concentration, 0.1 mM). Immunoprecipitation was performed in the presence of excess polyclonal antibodies to human apos (Boehringer
Levy: Role of Sar1b in lipid and lipoprotein production

Mannheim) at 4°C overnight. Samples were then washed with Nonidet P-40 (0.05%). They were subsequently centrifuged and resuspended in sample buffer (1.2% SDS, 12% glycerol, 60 mM Tris, pH 7.3, 1.2% β-mercaptoethanol, and 0.003% bromophenol blue) and analyzed by a linear 4–15% polyacrylamide gradient preceded by a 3% stacking gel. High Range Rainbow Molecular Weight Marker (Amersham Life Sciences) was run in the same conditions. Gels were sectioned into 2-mm slices and counted after an overnight incubation with 1 ml of Beckman tissue solubilizer (0.5 N quaternary ammonium hydroxide in toluene) and 10 ml of liquid scintillation fluid (Ready Organic, Beckman). Results for each apo studied were expressed as dpm/mg protein to assess the specific effect of Sar1b+/+ on apo synthesis and secretion.

**Lipid carrier**

Blood was drawn 3 h after the oral intake of a fat meal by a human volunteer, and postprandial plasma was prepared to serve as a carrier for the lipoproteins synthesized by Caco-2/15 cells. The TG-enriched plasma was incubated at 56°C for 1 h to inactivate enzymatic activity in the presence of antiproteases.

**Isolation of lipoproteins**

For the determination of secreted lipoproteins, Caco-2/15 cells were incubated with the lipid substrate as described above. The medium supplemented with antiproteases (as described above) was first mixed with a plasma lipid carrier (4:1, vol:vol) to efficiently isolate de novo lipoproteins synthesized. The lipoproteins were then isolated by sequential ultracentrifugation using a TL-100 ultracentrifuge (Beckman). Briefly, chylomicrons were isolated after ultracentrifugation (25,000 rpm for 40 min). Very low-density lipoprotein (1.006 g/ml) and low-density lipoprotein (LDL, 1.063 g/ml) were
Levy: Role of Sar1b in lipid and lipoprotein production

separated by spinning at 100,000 rpm for 2.30 h with a tabletop ultracentrifuge 100.4 rotor at 4°C. The high-density lipoprotein fraction was obtained by adjusting the LDL infranatant to density at 1.21 g/ml and centrifuging for 6.5 h at 100,000 rpm. Each lipoprotein fraction was exhaustively dialyzed against 0.15 M NaCl and 0.001 M EDTA, pH 7.0, at 4°C for 24 h.

**Microsomal triglyceride transfer protein activity assay (MTP)**

The MTP activity assay was performed on differentiated Caco-2/15 cells as described previously.1,5 Cells were plated at a density of 1x10^6 cells in 10 cm² plates in the same culture medium as above and differentiated for a period of 14 days. After collection, cells were resuspended in 10 mM phosphate-buffer containing 10 mg/ml saponin, 10 ug/ml of leupeptin and aprotinin and 1 ug/ml of pepstatin A. Cells were then centrifuged for 1 hour at 70000 rpm using a TL-100 ultracentrifuge (Beckman). Microsomal fraction was purified using a DEAE-cellulose (DE52-Whatman, Clifton, NJ) and eluted with a volume of 10 mM phosphate-buffer containing 220 mM NaCl. Microsomes were concentrated with a Centricon® cartridge (Millipore) at 5000 rpm for 25 minutes. MTP transfer activity was then measured by assessing radioactivity transfer between donor and acceptor unilamellar vesicles. The vesicles were prepared by mixing in 250 uL of chloroform the appropriate amounts of lipids. The donor vesicle contained, per assay, 4 nmol of 100 mg/mL phosphatidylcholine (Sigma), 0.33 nmol of 2 mg/mL cardiolipin (Sigma), 0.024 nmol of [3H]-trioleylglycerol (Amersham Biosciences), 0.01% BHT (Sigma) while the acceptor vesicle comprised 24 nmol of 100 mg/mL phosphatidylcholine, 0.048 nmol of trioleylglycerol, ~4000 cpm of [14C]-dipalmitoyl phosphatidylcholine ([14C]-DPPC; Amersham) and 0.01% BHT. The vesicles were then
Levy: Role of Sar1b in lipid and lipoprotein production

evaporated under a nitrogen stream, resuspended in a 15:35 buffer (15 mM Tris/HCl, pH 7.4, 35 mM NaCl, 1 mM EDTA, 3 mM NaN₃) containing 0.5% of free fatty acid-BSA and sonicated. Two different amounts of microsomal protein (5000 and 10000 ng) were incubated with 5 μL of each vesicle in 100 μL of 15:35 buffer without BSA and incubated for 1 h at 37°C. The transfer reaction was stopped by the addition of 400 μL of ice-cold 15:35 buffer with BSA. The donor vesicles were removed from the mixture by adsorption onto DEAE-cellulose at pH 7.4. The supernatant was collected after two centrifugations at 13000 g to ensure the complete removal of the donor vesicles-cellulose complex. The supernatant was then transferred to glass vials and radioactivity determined. Blank assay characterized by the incubation of both vesicles without protein served to determine the spontaneous exchange of radioactivity between the vesicles and was used to correct the percentage of lipid transfer. The ratio of [³H]-trioleylglycerol on [¹⁴C]-dipalmitoyl phosphatidylcholine was calculated, and the percentage lipid transfer was derived from the increase in this ratio.

**Monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) activity assays**

Differentiated Caco-2/15 cells were lysed in a buffer containing 50 mM Hepes, 250 mM sucrose, 1 mM EDTA, 50 mM NaF and 1 mM PMSF. MGAT and DGAT activities were assessed by measuring the incorporation of [¹⁴C] into diacylglycerol or TG following the incubation of 50 μM 2-oleoylglycerol or 250 μM 1,2-dioleoyl-sn-glycerol (both from Sigma) and 15-25 μM [¹⁴C]-palmitoyl-CoA (Amersham) in the presence of 15-25 μM cold palmitoyl-CoA (Sigma). Total lysates (25 μg) were incubated with the substrates in 100 mM Tris-HCl with 1 mg/mL BSA and 1 mM DTT (MGAT) or 16 mM MgCl₂.
Levy: Role of Sar1b in lipid and lipoprotein production

(DGAT) for 5 min at 23°C. After lipid extraction, lipid fractions were separated on TLC plates using the solvent mixture: hexane:ether:acetic acid, 60:40:4 (vol:vol:vol).

Process specificity

To determine the specificity of Sar1b, the transport of various nutrients such as 2-deoxy-D-[1-3H]-glucose, L-[3H]-glutamine and 45Calcium was examined. Transport was performed in differentiated Caco-2/15 cells that were serum starved 24 h prior to experiments. Measurements for 2-deoxy-D-[1-3H]-glucose (1 mM), L-[3H]-glutamine (0.5 mM) and 45Calcium (0.5 mM) was carried out for 10 min, 30 and 60 min. Cells were then washed three times each with ice-cold saline homogenized and counted in liquid-scintillation spectrometry.

Preparation of microsomes

Intestinal specimens were rinsed and homogenized (25%, w/v) in 0.25 M sucrose in buffer (50 mM Tris-HCl, pH 7.4, and 25 mM KCl) containing protease inhibitors (10 μg/ml leupeptin, 10 μg aprotinin, 1 μg/ml pepstatin, 1 μg/ml antipain, and 25 mM 4-aminophenyl-methanesulfonyl fluoride) using a polytron (Brinkmann Instruments) at a setting of 1 (30 s) and centrifuged for 15 min at 12,000 g at 4°C to prepare microsome fractions, a technique described earlier.1,7 The supernatant fraction was then centrifuged for 60 min at 100,000 g. The pellet was centrifuged for 60 min at 4°C. The washed microsomal pellets were quick frozen and stored at −80°C for later use.

Data analysis

To assess differences in the parameters studied, data were statistically analysed Student’s two tailed t-test. A p value <0.05 was considered statistically significant.
Table I: Presence and absence of proteins in endoplasmic reticulum-derived vesicles

<table>
<thead>
<tr>
<th>Classes of proteins tested</th>
<th>Detected proteins</th>
<th>Undetected proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolipoproteins</td>
<td>B-48</td>
<td>A-I, A-IV, E</td>
</tr>
<tr>
<td>COPII components</td>
<td>Sar1b, Sec12, Sec23, Sec 24</td>
<td>Sar1a, Sec13, Sec31</td>
</tr>
<tr>
<td>Fatty acid binding proteins</td>
<td>L-FABP</td>
<td>M-FABP, I-FABP</td>
</tr>
<tr>
<td>Cholesterol transporters</td>
<td>CD36</td>
<td>SR-BI, NPC1L1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>p125A, SCAP, SREBP-1c</td>
<td>PPAR(\gamma)</td>
</tr>
</tbody>
</table>

Endoplasmic reticulum-derived vesicles were isolated from Sar1b\(^{++}\) cells. Protein content was assessed by testing various classes using Western blotting.
REFERENCES


7. Levy E, Trudel K, Bendayan M, Seidman E, Delvin E, Elchebly M, Lavoie JC, Precourt LP, Amre D, Sinnett D. Biological role, protein expression, subcellular
SUPPLEMENTAL FIGURE LEGENDS

**Figure I**  **Cell integrity following Caco-2/15 cell transduction.** A series of studies were undertaken to determine whether the molecular manipulation procedure modified cell growth, differentiation and viability. The expression of villin (A) and sucrase (B), sensitive markers of cell differentiation, was not affected by lentivirus infection. Furthermore, no significant changes were noted in transepithelial resistance that represents an established measure of cellular monolayer integrity and stands for a qualitative assessment of the paracellular pathway aperture (C). Finally, cell viability by trypan blue exclusion was also assessed and was uniformly >98% (data not shown). All these parameters allowed us to conclude that Caco-2/15 cell transduction was not cytotoxic to Caco-2/15 cells and did not disrupt cell differentiation and viability as well as cell and membrane integrity. Results represent the means ± SEM of n=3 independent experiments.

**Figure II**  **Impact of Sar1b overexpression on lipid esterification.** Following transduction with lentivirus-\textit{Sar1b-GFP} and 14-d differentiation, Caco-2/15 cells were incubated with [\textsuperscript{14}C]-oleic acid for 20 h at 37°C. Lipids from cell homogenates and basolateral media were extracted with chloroform-methanol (2:1), separated by TLC and quantitated as described in MATERIALS AND METHODS. Results for, phospholipids (A) and cholesteryl ester (B) are expressed as dpm/mg cell protein, and values
represent means ± SEM of 6 separate experiments. *P<0.001, **P<0.0002, ***P<0.0001 vs control Mock cells.

**Figure III** Influence of Sar1b overexpression on apolipoprotein biosynthesis and secretion in Caco-2/15 cells. Following transduction with lentivirus-Sar1b-GFP and 14-d differentiation, Caco-2/15 cells were incubated with [³⁵S]-methionine in the apical compartment for 20 hours. Thereafter, cell homogenates and basolateral media were incubated overnight with a mixture of antibodies against apo B-100 (A), apo A-IV (B) and apo E (C) and Protein A/G agarose beads. The immune complexes were then thoroughly washed and analyzed by SDS-PAGE. After electrophoresis, gels were stained in Coomassie Blue and bands corresponding to each apo were excised and counted for the radioactivity incorporated. Data were analyzed as dpm/mg of total protein and represent means ± SEM for six independent experiments. *P<0.0001 vs control Mock cells.

**Figure IV** Impact of Sar1b overexpression on monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) activities in Caco-2/15 cells. Following transduction with lentivirus-Sar1b-GFP and 14-d differentiation, Caco-2/15 cells were differentiated for a period of 14 days and microsomes were prepared and assayed for the two enzymes. Data were expressed as µmol/min/mg protein and represent the means ± SEM for n=6 independent experiments. *P<0.001, **P<0.003 vs control Mock cells.
Figure V  **Sar1a protein status in Caco-2/15 cells overexpressing Sar1b.** Sar1b gene expression was forced as described in Materials and Methods. Thereafter, cellular proteins were resolved by SDS-PAGE and immunoblotted for Sar1a and β-actin. Representative immunoblots are documented.
Figure I

A

Villin/β-actin

B

Sucrase/β-actin

C

Mock

Sar1b^{+/+}

Ohm/cm^2
Figure II

**Mock**

<table>
<thead>
<tr>
<th></th>
<th>Cells</th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>dpm/mg protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mock</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sar1b^{+/+}</strong></td>
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</tbody>
</table>

A

B

**Significance:**

- ***: p < 0.001
- **: p < 0.01
- *: p < 0.05
Figure III

A

B

C

Mock

Sar1b^{+/+}

dpm/mg protein

Cells
Media
Total

Cells
Media
Total

Cells
Media
Total

Cells
Media
Total

*
Figure IV

A

B

μmoles/min/mg protein

Mock

Sar1b+/+

μmoles/min/mg protein

* **
Figure V

Mock

Sar1a

β-actin

Sar1a/β-actin

0.0

0.2

0.4

Mock

Sar1b^{+/+}

0.0

0.2

0.4