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The Molecular Basis of Retinoid Absorption

*A GENETIC DISSECTION**

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The intestine and other tissues are able to synthesize retinyl esters in an acyl-CoA-dependent manner involving an acyl-CoA:retinol acyltransferase (ARAT). However, the molecular identity of this ARAT has not been established. Recent studies of lecithin:retinol acyltransferase (LRAT)-deficient mice indicate that LRAT is responsible for the preponderance of retinyl ester synthesis in the body, aside from in the intestine and adipose tissue. Our present studies, employing a number of mutant mouse models, identify diacylglycerol acyltransferase 1 (DGAT1) as an important intestinal ARAT *in vivo*. The contribution that DGAT1 makes to intestinal retinyl ester synthesis becomes greater when a large pharmacologic dose of retinol is administered by gavage to mice. Moreover, when large retinol doses are administered another intestinal enzyme(s) with ARAT activity becomes apparent. Surprisingly, although DGAT1 is expressed in adipose tissue, DGAT1 does not catalyze retinyl ester synthesis in adipose tissue *in vivo*. Our data also establish that cellular retinol-binding protein, type II (CRBPII), which is expressed solely in the adult intestine, *in vivo* channels retinol to LRAT for retinyl ester synthesis. Contrary to what has been proposed in the literature based on *in vitro* studies, CRBPII does not directly prevent retinol from being acted upon by DGAT1 or other intestinal ARATs *in vivo*.

Retinoids (vitamin A and its natural and synthetic analogs) are required for maintaining many essential physiological functions in vertebrates, including growth and differentiation, reproduction, embryogenesis, immune function, and vision (1–3). All retinoids must originate from the diet either as preformed retinoid or as proretinoid carotenoids (4). The preformed retinoid is taken up from the intestinal lumen into mucosa cells as the free alcohol retinol. Proretinoid carotenoids like β-carotene are absorbed by the mucosa intact and enzymatically converted to retinaldehyde and subsequently to retinol within the cell. Within enterocytes, retinol is esterified with long-chain fatty acyl groups to form retinyl esters, which are packaged along with dietary lipids into nascent chylomicrons (4–6). In healthy animals and humans, ~66–75% of the chylomicron remnant retinoid is cleared by the liver (4–6). The remainder is cleared by extrahepatic tissues, including adipose tissue, lung, muscle, and to lesser extents by other tissues.

We are interested in elucidating the molecular processes that are important for facilitating retinoid absorption by the intestine. Several proteins are known to play important roles in this process. Lecithin:retinol acyltransferase (LRAT),[2] which catalyzes the transesterification of retinol with a fatty acyl group from the *sn*-1 position of membrane lecithin as an acyl donor, has long been proposed to have an important role in retinoid absorption (7–10). Studies of *Lrat*-deficient ($Lrat^{-/-}$) mice indicate that they have only 50–60% of the intestinal absorption efficiency of wild type mice when challenged with a physiologic dose of retinol (11). Moreover, unlike wild type mice, ~60% of the retinoid absorbed in chylomicrons of $Lrat^{-/-}$ mice is as unesterified retinol, and not as retinyl ester (11). Based on *in vitro* evidence, the literature suggests that an acyl-CoA-dependent enzyme, referred to as acyl-CoA:retinol acyltransferase (ARAT), may also have a role in catalyzing retinyl ester formation within the intestine (12–14). Yen *et al.* (15), Orland *et al.* (16), and O'Byrne *et al.* (11) have shown through *in vitro* assays that the enzyme diacylglycerol acyltransferase 1 (DGAT1) is able to esterify retinol to retinyl esters in an acyl-CoA-dependent manner. However, it is not clear whether DGAT1 has a physiological role in catalyzing retinyl ester formation *in vivo*. Because retinol is very hydrophobic, it is normally bound specifically to cellular retinol-binding proteins (CRBPs) within cells (17–18). One of the proposed physiologic roles of the CRBPs is the metabolic channeling of retinol toward enzymes that use it as a substrate (10, 17–20). Based on *in vitro* studies, it has been proposed that LRAT utilizes preferentially retinol bound to CRBPI (expressed in most adult tissues with the exception of the small intestine) or retinol bound to CRBPII (expressed solely in the adult intestine) as substrates rather

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[2] The abbreviations used are: LRAT, lecithin:retinol acyltransferase; ARAT, acyl-CoA:retinol acyltransferase; ATGL, adipose triglyceride lipase; CRBPI, cellular retinol-binding protein, type I; CRBPII, cellular retinol-binding protein, type II; $Crbpll^{-/-}$ mice, *Crbpll*-deficient mice; CRBPIII, cellular retinol-binding protein, type III; DGAT1, diacylglycerol acyltransferase 1; DGAT2, diacylglycerol acyltransferase 2; $Dgat1^{-/-}$ mice, *Dgat1*-deficient mice; HPLC, high performance liquid chromatography; HSL, hormone-sensitive lipase; $Lrat^{-/-}$ mice, *Lrat*-deficient mice; RBP, retinol-binding protein.

than unbound retinol. This is in contrast to ARAT, which based on *in vitro* studies is incapable of esterifying retinol when it is bound to CRBPI or CRBPII (10, 17–20).

To understand better the molecular processes mediating the uptake of retinoid postprandially into the circulation, we employed a number of lines of knock-out mice to investigate these processes. We used previously described *Lrat*$^{-/-}$ (21), *CrbpII*$^{-/-}$ (22), and *Dgat1*$^{-/-}$ (23) single knock-out mice as well as *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ double knock-out mice that we generated for these studies. We have used these mouse models to gain new insight into several questions about the biochemical mechanisms that are important for mediating uptake and processing of dietary retinoid from the intestine. These questions include the following. 1) Is DGAT1 a physiologically and/or pharmacologically important intestinal ARAT? 2) Are there other physiologically important ARATs in the body? 3) *In vivo*, does CRBPII channel retinol to LRAT and/or directly prevent retinol from being acted upon by intestinal ARATs?

EXPERIMENTAL PROCEDURES

Animals, Animal Husbandry, and Diets—The mutant mouse lines involving single gene disruptions that we employed in our studies all have been described in the literature and include *Lrat*$^{-/-}$ (21), *CrbpII*$^{-/-}$ (22), and *Dgat1*$^{-/-}$ (23) mice. *Lrat*$^{-/-}$ mice were in a mixed C57Bl/6J/129sv genetic background. *CrbpII*$^{-/-}$ and *Dgat1*$^{-/-}$ mice were in the C57B1/6J genetic background. Using conventional breeding protocols we also generated several mutant mouse lines that harbor two disrupted genes. These include *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice. Progeny obtained from crosses of heterozygotes were used in all of our studies. Genotypes of the mice were determined by published PCR protocols using tail clip DNA as described in the literature (21–23). Routinely, animals were allowed *ad libitum* access to water and a standard nutritionally complete rodent chow diet (W. F. Fisher and Sons, Inc.). All mice were maintained on a 12-h dark-light cycle, with the period of darkness between 7:00 a.m. and 7:00 p.m. in a conventional barrier facility. For all of our studies, male and/or female mice at 3 months of age were employed and sacrificed in the morning between 9:30 and 11:30 am. The animal experiments described in this report were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Columbia University Institutional Committee on Animal Care.

Intestinal Retinoid Absorption—These investigations were carried out as described previously for our study of *Lrat*$^{-/-}$ mice (11). Briefly, a gavage dose consisting of either 6 μg of retinol and 1 μCi of [11,12-^{3}H]retinol (49.3 Ci/mmol, PerkinElmer Life Sciences) (physiological dose) or 1,000 μg of retinol (pharmacological dose) was prepared in 50 μl of peanut oil (6 μg of retinol containing $\sim 1 \times 10^6$ cpm/50-μl dose or 1,000 μg of retinol but containing no tracer/50-μl dose, respectively). Three-month-old male wild type, *Dgat1*$^{-/-}$, *Lrat*$^{-/-}$, *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$, *CrbpII*$^{-/-}$, and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ mice were used for these studies. After an overnight fast and treatment with the lipase inhibitor, P-407 (1 g/kg body weight) administered by intraperitoneal injection (24, 25), mice were given an oral bolus dose in 50 μl of peanut oil containing either a physiological dose or a pharmacological dose of retinol. Mice were sacrificed 4 h after dose administration, and serum and the intestine were collected for analysis. To study the distribution of newly absorbed retinoid in the chylomicron fraction, serum samples from individual mice were pooled according to gender and genotype (3–5 mice per pool). These pools (containing $\sim$1.5 ml of serum) were overlaid with $\sim$10 ml of PBS (PBS, 10 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride) in an ultracentrifuge tube and centrifuged in an SW 40 rotor at 20 °C for 1 h at 39,000 rpm in a Beckman L8M ultracentrifuge. The chylomicrons floating at the top of the PBS layer were aspirated and washed again with PBS to minimize the contamination by a second centrifugation at the same conditions. Washed chylomicrons were used in our analyses. To assess the total [^{3}H]retinoid in the washed chylomicrons, 10 μl of each washed chylomicron fraction was transferred to a scintillation vial and dissolved in 10 ml of Hydroflor liquid scintillation counting solution (National Diagnostics). To assess individual retinoids present in the washed chylomicrons, the chylomicron fractions were extracted and analyzed by reverse phase HPLC, exactly as described below for tissue homogenates. Fractions were collected at 0.5-min intervals throughout the entire HPLC run, and [^{3}H]retinoid for each fraction was determined as described above. The ^{3}H counts/min present in the fractions were measured in a Beckman LS 1800 liquid scintillation counter. For the pharmacological doses of retinol, each blood sample was collected, allowed to coagulate for 1 h on ice, and centrifuged at 4 °C to obtain serum. To determine retinoid contents present in the serum, the serum was extracted and analyzed by reverse phase HPLC as described below for tissue homogenates. Serum triglyceride levels were assessed using a kit following exactly the manufacturer's instructions (Thermo Fisher Scientific). To assess the proportion of the retinol dose (as either retinol or retinyl ester) remaining associated with the intestine after sacrifice of the mice, the first 5 cm of the small intestine was dissected and longitudinally opened. The intestine was then briefly immersed in a beaker containing PBS and subsequently blotted gently with absorbent paper. The washed intestine was then weighed, homogenized, and analyzed as described below under HPLC analysis.

HPLC Analysis—Tissue and serum retinol and retinyl ester levels were determined by procedures described previously (11). Briefly, serum, liver, intestine (consisting of the 5 cm most proximal to the stomach), and perigonadal (epididymal or ovarian) adipose tissue were flash-frozen in liquid N_2 after dissection from the mice and stored immediately at −80 °C prior to analysis. Tissues were homogenized in 10 volumes of PBS (10 mM sodium phosphate, pH 7.2, 150 mM sodium chloride) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) set at half-maximal speed for 10 s. An aliquot of serum or tissue homogenate (200-μl aliquots of the total homogenate) was then treated with an equal volume of absolute ethanol containing a known amount of retinyl acetate as an internal standard, and the retinoids present in the homogenates were extracted into hexane. The extracted retinoids were separated on a 4.6 × 250 mm Ultrasphere C_{18} column (Beckman, Fullerton, CA) preceded by a C_{18} guard column (Supelco, Bellefonte,

PA), using 70% acetonitrile, 15% methanol, 15% methylene chloride as the running solvent flowing at 1.8 ml/min. Retinol and retinyl esters (retinyl palmitate, oleate, linoleate, and stearate) were detected at 325 nm and identified by comparing the retention times and spectral data of experimental compounds with those of authentic standards. Concentrations of retinol and retinyl esters in the tissues were quantitated by comparing integrated peak areas for those of each retinoid against those of known amounts of purified standards. Loss during extraction was accounted for by adjusting for the recovery of the internal standard added immediately after homogenization of the tissues.

Retinoic Acid Formation by Intestine Mucosal Scrapings—To assess the capacity of the intestinal mucosa of wild type and *CrbpII*$^{-/-}$ mice to synthesize retinoic acid from retinol or retinaldehyde, we employed experimental conditions previously described by Dew and Ong (26). For this purpose 3-month-old male wild type and *CrbpII*$^{-/-}$ mice were fasted overnight. Sections of small intestine, 5 cm in length, most proximal to the stomach were dissected and longitudinally opened. The dissected material was washed with ice-cold Krebs-Ringer phosphate buffer (20 mM sodium phosphate, 125 mM NaCl, 4.93 mM KCl, 1.23 mM $MgSO_4$, 0.85 mM $CaCl_2$, and 10 mM glucose, pH 6.5) and gently blotted with absorbent paper to remove excess buffer. Mucosa cells were scraped from the luminal surface using a clean glass microscope slide, pooled for 4–5 mice of each strain, and placed into incubation buffer (Krebs-Ringer buffer containing taurocholate at a final concentration of 0.06 μM). Protein concentrations of mucosal scraping were determined by using Bio-Rad DC protein assay kit according to the supplier's instructions. Retinol or retinaldehyde prepared in ethanol was added to mucosal scrapings so that the molar concentration of retinoid would be 50% of the concentration of CRBPII expected to be present in the wild type scrapings. The calculation of CRBPII concentration was based on the reported finding that CRBPII is present as 0.4–1% of total cytosolic protein in the small intestine (10, 27). After adding retinol or retinaldehyde, the mixtures were incubated at 37 °C for 1 h in a shaking incubator. Subsequent retinoic acid determinations were carried out by normal phase HPLC using procedures we have described earlier (11).

In Vitro Assays of Retinyl Ester Formation—To assess the possibility that diacylglycerol acyltransferase 2 (DGAT2), hormone-sensitive lipase (HSL), or adipose triglyceride lipase (ATGL) is able catalyze retinyl ester formation from retinol, we incubated whole cell homogenates prepared from McA-RH777 hepatocytes expressing either human DGAT2, HSL, ATGL, or an empty vector with retinol (200 μM, final concentration) for 1 or 2 h at 37 °C. The McA-RH777 homogenates were a kind gift of Dr. Li-Shin Huang of the Department of Medicine, Columbia University. The assay buffer consisted of 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 1 mM EDTA. The cells were homogenized in assay buffer supplemented with 0.2% fatty acid-free bovine serum albumin and 1 mM dithiothreitol. The assay was stopped through addition of an equal volume of 100% ethanol, and the retinoids were extracted into hexane. The resulting retinoid-containing hexane extract was analyzed by reverse phase HPLC as described above. Homogenates of cells expressing DGAT2 were able to catalyze triglyceride formation from exogenous diacylglycerol (25 μM, final concentration) and oleoyl-CoA (50 μM, final concentration). Similarly, homogenates expressing HSL or ATGL were catalytically active in catalyzing triglyceride hydrolysis (1.25 mM final triolein concentration). The homogenates of McA-RH777 cells expressing the empty vector were unable to catalyze either triglyceride formation or triglyceride/diglyceride hydrolysis.

Statistical Analyses—All data were analyzed for statistically significant differences using standard procedures consisting of an unpaired *t* test or an analysis of variance followed by pairwise comparisons.

RESULTS

DGAT1 Catalyzes Intestinal Retinyl Ester Formation in Vivo—Previous studies demonstrated that intestinal retinyl ester synthesis is markedly impaired but not completely abolished in *Lrat*$^{-/-}$ mice (11). The synthesis of retinyl ester by *Lrat*$^{-/-}$ mice in response to a gavage dose of a physiological amount of retinol was less than 10% that of wild type mice given the same dose. The retinyl ester composition observed for chylomicrons isolated from *Lrat*$^{-/-}$ mice suggested that for these mice intestinal retinyl ester formation involves an acyl-CoA-dependent enzyme or an ARAT. DGAT1, an enzyme that is expressed by the intestine and catalyzes acyl-CoA-dependent formation of triglyceride from diglyceride, is able to catalyze acyl-CoA-dependent esterification of retinol *in vitro* (11, 15, 16). To assess whether DGAT1 is responsible for intestinal synthesis of retinyl ester in *Lrat*$^{-/-}$ mice, we generated *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice. These mutants were viable, and at 3 months of age showed no visible abnormalities. We investigated whether *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice were able to esterify retinol to retinyl esters in response to a physiological challenge of retinol (6 μg containing 1 μCi of [^{3}H]retinol; this is approximately the amount of retinol consumed by a mouse in 1 day when maintained on a standard rodent chow diet). For all of our studies, we employed the same experimental protocols that we used in our original investigations of *Lrat*$^{-/-}$ mice, including the use of the total lipase inhibitor P-407 to inhibit clearance of postprandial lipids, including retinoid, from the circulation (11, 24, 25) (see "Experimental Procedures"). Consequently, our measure of postprandial retinoid levels provides an assessment of the total retinoid absorbed over the 4-h period after administration of a gavage dose of retinol in peanut oil. From fractions collected upon HPLC analysis, we detected retinyl esters in chylomicrons obtained from wild type, *Dgat1*$^{-/-}$, and *Lrat*$^{-/-}$ mice, but not from *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice (Fig. 1). For both *Lrat*$^{-/-}$ and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice compared with wild type and *Dgat1*$^{-/-}$ mice, we detected elevated levels of unesterified retinol in the isolated chylomicron fractions. For *Lrat*$^{-/-}$ mice, the majority of the ^{3}H counts/min and retinoid mass appeared in the fractions corresponding to free retinol (Fig. 1, *panel C*) with a lesser amount of ^{3}H counts/min and retinoid mass present as retinyl ester (primarily as retinyl linoleate and retinyl oleate). For *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice, we were able to detect only ^{3}H counts/min and retinoid mass corresponding to free (unesterified) retinol (Fig. 1, *panel D*). This same result was obtained from three independent experiments. Serum triglyc-

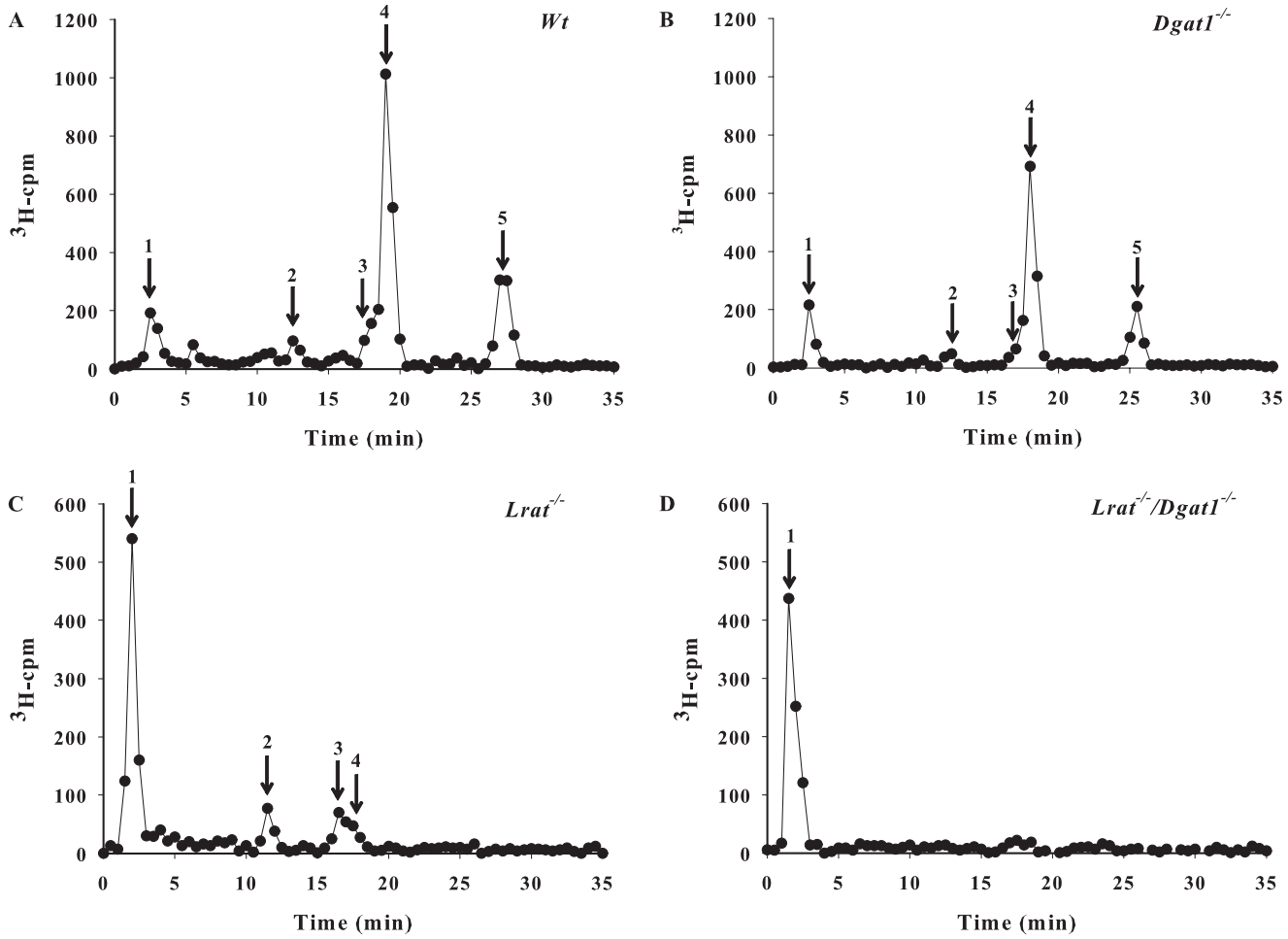

FIGURE 1. **DGAT1 can catalyze intestinal retinyl ester synthesis *in vivo*.** Distribution of [^{3}H]retinoids present in washed chylomicrons were obtained from P-407-treated wild type (*Wt*) (*panel A*), *Dgat1*$^{-/-}$ (*panel B*), *Lrat*$^{-/-}$ (*panel C*), and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ (*panel D*) mice 4 h after administration by gavage of a physiological dose of retinol (6 μg of retinol containing 1 $\times$ 10^6 ^{3}H counts/min) in 50 μl of peanut oil. Extracted retinoids were separated on a 5-μm 4.6 $\times$ 250-mm Ultrasphere C_{18} column preceded by a C_{18} guard column, using 70:15:15 (v/v) acetonitrile/methanol/methylene chloride as the running solvent flowing at 1.8 ml/min. The *numbers above* the HPLC peaks indicate the following: *1*, retinol; *2*, retinyl linoleate; *3*, retinyl oleate; *4*, retinyl palmitate; and *5*, retinyl stearate.

eride levels, measured in response to the gavage dose of retinol given in peanut oil, were not different for wild type, *Dgat1*$^{-/-}$, *Lrat*$^{-/-}$, and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice. Moreover, when we examined the proximal 5 cm of the small intestines for their residual retinoid content, we did not observe statistically significant differences in the amount of [^{3}H]retinoid that remained associated with the intestine. We take these data collectively to indicate that, in the absence of LRAT, intestinal DGAT1 acts *in vivo* as an ARAT in response to a physiological challenge of retinol.

To characterize more extensively the *Dgat1*$^{-/-}$ mice with regard to normal retinoid physiology, we assessed the capacity of *Dgat1*$^{-/-}$ mice for retinoid absorption and storage. Like wild type mice, *Dgat1*$^{-/-}$ mice, in response to a challenge with a physiological dose of retinol (6 μg), showed abundant retinyl esters in the postprandial circulation, primarily as retinyl palmitate and retinyl stearate along with low concentrations of retinyl linoleate and retinyl oleate (Fig. 1, *panel B*). Moreover, we were not able to detect statistically significant differences in the amount of either retinol (1.1 $\pm$ 0.1 μM for wild type *versus* 1.2 $\pm$ 0.2 μM for *Dgat1*$^{-/-}$ mice) or retinyl ester (2.7 $\pm$ 0.3 μM for wild type *versus* 3.0 $\pm$ 0.4 μM for *Dgat1*$^{-/-}$ mice) in the postprandial circulations of P-407-treated wild type and *Dgat1*$^{-/-}$ mice upon challenge with a physiological dose of retinol. Hepatic total retinol (retinol + retinyl ester) contents also were not statistically different for age-, diet-, and gender-matched wild type and *Dgat1*$^{-/-}$ mice (Table 1). These data are consistent with the notion that in times of normal dietary retinoid intake LRAT accounts for the preponderance of retinyl ester formation by the intestine.

TABLE 1

Hepatic retinol and retinyl ester concentrations for 3-month-old male wild type and *Dgat1*$^{-/-}$ mice maintained on a control chow diet since the time of weaning

All values are given as means $\pm$ 1 S.D.

Strain	Retinol	Retinyl ester	Total retinol
	nmol/g liver	*nmol/g liver*	*nmol/g liver*
Wild type (n = 21)	151.1 $\pm$ 55.4	2763.1 $\pm$ 933.1	2914.1 $\pm$ 972.5
Dgat1$^{-/-}$ (n = 13)	201.2 $\pm$ 41.8	2885.2 $\pm$ 670.6	3085.4 $\pm$ 687.9

ARAT activity has been hypothesized in the literature to be importantly involved in processing pharmacological doses of retinol (12, 20, 28). To explore whether this activity was because

of DGAT1, a pharmacological dose of retinol (1,000 μg of unlabeled all-*trans*-retinol or approximately the amount of retinol that is consumed by a mouse in 6 months when maintained on a standard rodent chow diet) was administered to age- and gender-matched wild type, *Lrat*$^{-/-}$, *Dgat1*$^{-/-}$, and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ littermates, and the accumulation of retinoid in the postprandial circulation was assessed. Four h after dose administration, mice were sacrificed, and serum was analyzed by HPLC. Both retinol and retinyl ester levels were markedly increased in *Lrat*$^{-/-}$ mice given a pharmacological dose of retinol compared with those given a physiological dose of retinol. Serum from *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice given a pharmacological dose of retinol also showed quantitatively greater retinol levels compared with *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice given the physiological dose of retinol. Moreover, unlike the case for the physiologic dose of retinol, retinyl ester was present in the postprandial circulation of the *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice given the pharmacologic retinol dose. Greater than 90% of retinoid present in the serum of wild type and *Dgat1*$^{-/-}$ mice given the pharmacologic dose was present as retinyl ester, whereas less than 40% of retinoid was detected in the serum of *Lrat*$^{-/-}$ mice and much less still for the *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice was present as retinyl ester (Fig. 2, *panels A* and *B*). Although *Dgat1*$^{-/-}$ mice showed no differences when compared with wild type mice in intestinal absorption in response to a physiological challenge of retinol, these mice were ~70% less efficient than wild type mice in absorbing a large pharmacologic dose of retinol. Fig. 2, *panel B,* shows that retinyl ester levels are significantly lower in postprandial serum of *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice compared with those present from *Lrat*$^{-/-}$ or *Dgat1*$^{-/-}$ mice given the same pharmacologic dose of retinol. Interestingly, as seen in Fig. 2, *panel A*, the levels of retinol absorbed by *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice are also significantly lower than those for *Lrat*$^{-/-}$ mice but still elevated when compared statistically to those of wild type or *Dgat1*$^{-/-}$ mice These data convincingly establish a role for DGAT1 in intestinal retinol esterification.

Because LRAT catalyzes the transesterification of retinol with an acyl group removed from the *sn*-1 position of membrane lecithin (7–10), a position where the most abundant acyl groups are saturated fatty acids (primarily esters of palmitic acid or stearic acid), retinyl palmitate and retinyl stearate are the most abundant retinyl esters arising from LRAT action. Retinyl linoleate and retinyl oleate are less frequently formed by LRAT action and appear to arise primarily through ARAT action. Because of this difference in acyl group utilization, we calculated a PS/LO ratio ((moles of retinyl palmitate + moles of retinyl stearate)/(moles of retinyl linoleate + moles of retinyl oleate)) for circulating postprandial retinyl esters to serve as an index of LRAT *versus* ARAT activities for all of the mouse strains studied. As evidenced in Fig. 2, *panel C*, wild type and *Dgat1*$^{-/-}$ mice show a high PS/LO ratio indicating that for these mice LRAT was primarily responsible for catalyzing intestinal retinol esterification. However, for *Lrat*$^{-/-}$ and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice, the ratio of PS/LO was significantly lower than wild type and *Dgat1*$^{-/-}$ mice indicating that the other enzyme(s) responsible for retinyl ester formation in the intestine in response to a pharmacological dose of retinol also acts through an acyl-CoA-dependent or ARAT mechanism.

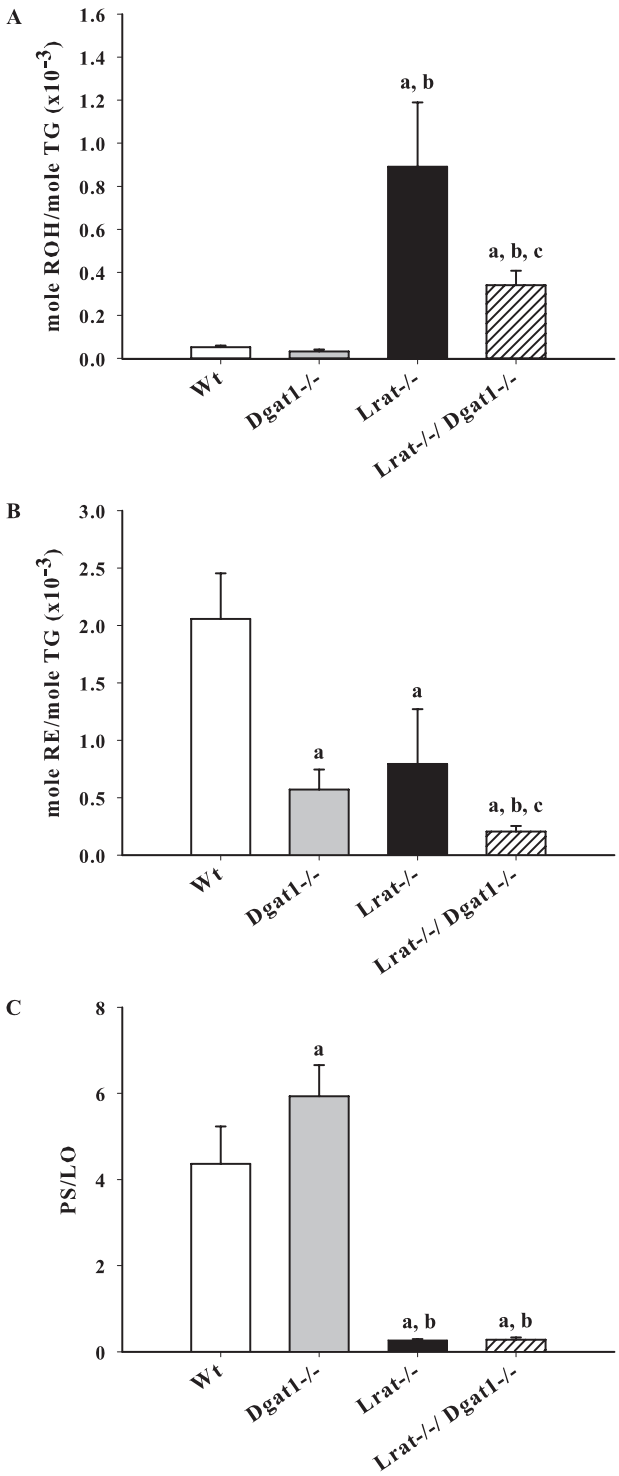

FIGURE 2. **The contribution made by DGAT1 toward intestinal retinyl ester synthesis becomes greater in response to a pharmacologic dose of retinol.** Retinol levels (*panel A*), retinyl ester levels (*panel B*), PS/LO ratio (*panel C*) in postprandial serum were obtained from P-407-treated male wild type (*Wt*) ($n = 5$), *Dgat1*$^{-/-}$ ($n = 8$), *Lrat*$^{-/-}$ ($n = 6$), and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ ($n = 13$) littermates 4 h after administration by gavage of a pharmacological dose of retinol (1,000 μg of retinol) in 50 μl of peanut oil. Retinol (*ROH*) and retinyl ester (*RE*) levels are normalized to plasma triglyceride (*TG*) levels. All values are given as means $\pm$ 1 S.D. The PS/LO ratio represents ((moles of retinyl palmitate + moles of retinyl stearate)/(moles of retinyl linoleate + moles of retinyl oleate). Statistical significance is as follows: *a*, $p < 0.01$ compared with wild type mice; *b*, $p < 0.01$ compared with *Dgat1*$^{-/-}$ mice; and *c*, $p < 0.01$ compared with *Lrat*$^{-/-}$ mice.

TABLE 2

Retinol and retinyl ester levels in adipose tissue obtained from 3-month-old male and female wild type, *Dgat1*$^{-/-}$, *Lrat*$^{-/-}$, and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ littermates

All values are given as means ± 1 S.D. The number of individual mice (*n*) used for each measurement is given in the parentheses.

Strain	Gender	Retinol	Retinyl ester	Total retinol
		nmol/g tissue wet weight	*nmol/g tissue wet weight*	*nmol/g tissue wet weight*
Wild type	Male (8)	3.1 ± 0.9	9.7 ± 2.5	12.8 ± 3.0
	Female (3)	2.5 ± 0.3	6.7 ± 1.0	9.2 ± 0.9
Dgat1$^{-/-}$	Male (4)	2.0 ± 0.5	9.5 ± 5.2	11.5 ± 5.5
	Female (3)	2.2 ± 0.3	9.3 ± 2.3	11.5 ± 2.6
Lrat$^{-/-}$	Male (12)	7.1 ± 3.1[a]	27.2 ± 12.0[a]	34.2 ± 11.3[a]
	Female (7)	6.7 ± 3.5	25.3 ± 6.2[a]	31.9 ± 7.1[a]
Lrat$^{-/-}$/*Dgat1*$^{-/-}$	Male (13)	5.4 ± 3.4	22.5 ± 11.8[a]	27.9 ± 14.4[a]
	Female (15)	5.7 ± 4.4	21.0 ± 9.3[a]	26.7 ± 11.6[a]

[a] The statistical significance is $p < 0.01$ compared with gender-matched wild type mice.

DGAT1 Is Not Needed for Catalyzing Retinyl Ester Formation in Adipose Tissue—Only trace amounts of retinyl esters were detected in liver, eyes, lungs, testes, and kidney of *Lrat*$^{-/-}$ mice (11, 21, 29). Adipose tissue however contained substantial retinyl ester stores in *Lrat*$^{-/-}$ mice (11, 29). In fact, adipose retinyl ester levels were elevated in *Lrat*$^{-/-}$ mice suggesting the existence of a compensatory pathway for retinoid accumulation in this tissue. Because *Dgat1* is highly expressed in adipose tissue (30), we investigated the importance of *Dgat1* for catalyzing retinol esterification in adipose tissue, and we determined adipose tissue retinyl ester levels in *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice and compared these with wild type, *Dgat1*$^{-/-}$, and *Lrat*$^{-/-}$ littermates. If DGAT1 is a significant enzyme for catalyzing retinyl ester formation in adipose tissue, then retinyl ester levels in adipose tissue should be diminished in *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice. Table 2 shows retinol and retinyl ester concentrations for adipose tissue from 3-month-old male and female wild type, *Dgat1*$^{-/-}$, *Lrat*$^{-/-}$, and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ littermates maintained on a standard chow diet since weaning. Both retinol and retinyl ester levels were elevated 2–3-fold in *Lrat*$^{-/-}$ mice compared with wild type mice for both males and females. There were no significant differences in adipose levels of either retinol or retinyl ester in *Dgat1*$^{-/-}$ mice compared with age-, gender-, and diet-matched wild type mice. However, adipose retinol and retinyl ester levels for *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice were statistically elevated over those of wild type mice but statistically identical to those of their *Lrat*$^{-/-}$ littermates.

We asked whether several other lipid-metabolizing enzymes expressed in adipose tissue might be able to catalyze retinyl ester formation from retinol. McA-RH777 cell homogenates harboring catalytically active recombinant DGAT2, which like DGAT1 is expressed in adipose tissue and is able to catalyze acyl-CoA-dependent triglyceride formation (31), were unable to catalyze retinyl ester formation when retinol and palmitoyl-CoA were added to incubation mixtures. This finding regarding DGAT2 is in agreement with that of Yen *et al.* (15). We were not able to demonstrate *in vitro* that recombinant ATGL, which has been reported to catalyze transesterifications of acyl groups between acylglycerol species (32), can catalyze retinyl ester formation from retinol. Recombinant HSL, which catalyzes retinyl ester hydrolysis (33), similarly was unable to catalyze retinyl ester formation from retinol.

Role of CRBPII in Retinoid Absorption—Observations from many *in vitro* studies have led to the hypotheses that CRBPII-bound retinol is metabolically channeled to LRAT and is directly prevented from being utilized by intestinal ARAT(s)

TABLE 3

Retinol and retinyl ester (RE) levels and PS/LO ratio in postprandial serum obtained from P-407-treated male wild type and *CrbpII*$^{-/-}$ mice upon administration of either a physiologic dose or pharmacologic dose of retinol in peanut oil

All values are given as means ± 1 S.D.

Strain	Retinol	Retinyl ester	PS/LO
	(mole ROH or RE/mole triglyceride) × 10^{-6}		
Physiologic dose			
Wild type ($n = 5$)	4.6 ± 0.6	18.3 ± 12.0	12.8 ± 3.5
CrbpII$^{-/-}$ ($n = 5$)	4.0 ± 0.6	27.0 ± 6.2	9.8 ± 1.9
	(mole ROH or RE/mole triglyceride) × 10^{-3}		
Pharmacologic dose			
Wild type ($n = 12$)	0.1 ± 0.0	2.4 ± 0.9	4.6 ± 1.5
CrbpII$^{-/-}$ ($n = 22$)	0.1 ± 0.1	2.0 ± 0.6	3.6 ± 0.7[a]

[a] The statistical significance is $p < 0.02$ compared with wild type mice receiving the same dose.

(10, 17). *CrbpII*$^{-/-}$ mice generated by Li and co-workers (22) have been described, but these mice have not been extensively studied with regard to these possibilities. To explore these hypotheses *in vivo*, we used the same experimental protocols as used above in our studies of *Lrat*$^{-/-}$, *Dgat1*$^{-/-}$, and *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice. We administered by gavage doses of retinol, both physiological (6 μg) and pharmacological (1,000 μg) doses, to P-407-treated wild type and *CrbpII*$^{-/-}$ mice. For a physiologic dose of retinol, we did not observe a statistically significant difference in either the amount of retinol or retinyl ester present in the postprandial circulations of wild type and *CrbpII*$^{-/-}$ mice (Table 3). Nor, at the time of sacrifice, did we observe any difference in the amount of residual retinoid dose associated with the proximal 5 cm of the small intestine from *CrbpII*$^{-/-}$ (1.6 ± 0.2% of dose, $n = 5$) compared with wild type mice (1.3 ± 0.6% of dose, $n = 5$), suggesting that the residence time of the retinoid dose in the intestine was not altered by the absence of CRBPII. As seen in Table 3 there was a trend toward a lower mean PS/LO ratio for *CrbpII*$^{-/-}$ mice, but this trend was not statistically significant. For mice receiving a pharmacologic dose of retinol, we also did not observe differences in the amounts of either retinol or retinyl ester taken up into the postprandial circulations of wild type and *CrbpII*$^{-/-}$ mice (Table 3). However, unlike for the physiologic retinol dose, the PS/LO ratio calculated for postprandial retinyl ester was statistically lower ($p < 0.02$) in *CrbpII*$^{-/-}$ mice compared with wild type mice. Interestingly, the PS/LO ratio for wild type mice receiving the pharmacologic dose of retinol was substantially lower than that obtained for wild type mice receiving the physiological dose. This suggests an increased involvement of intestinal

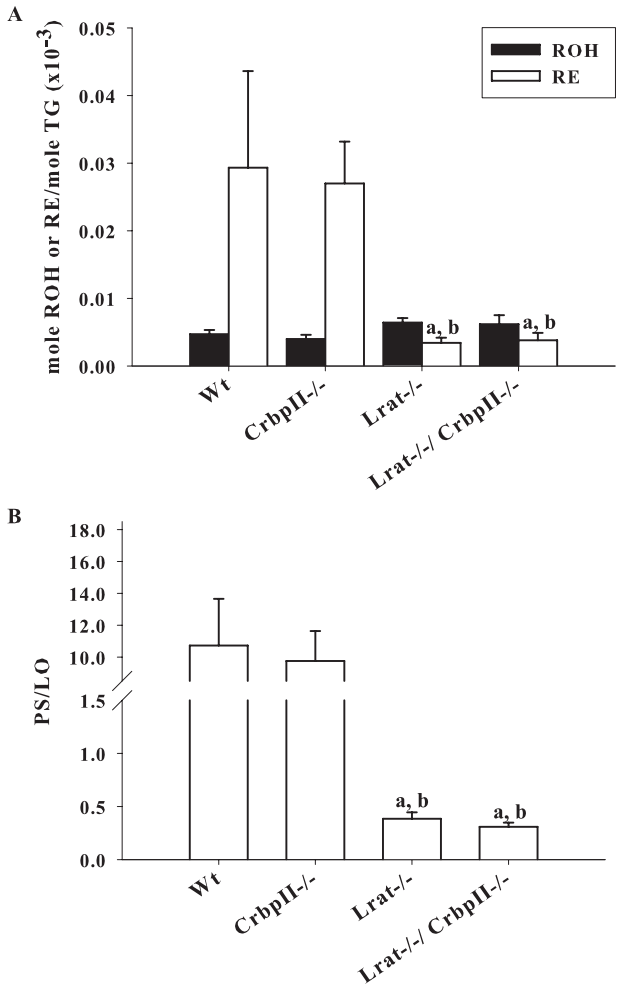

FIGURE 3. **The effect of CRBPII absence on the amount of retinol taken up by the intestine in response to a physiologic challenge with retinol.** Retinol (*black bars*) and retinyl ester (*open bars*) levels (*panel A*) and PS/LO ratio (*panel B*) in postprandial serum obtained from P-407-treated male wild type (*Wt*) ($n = 9$), *CrbpII*$^{-/-}$ ($n = 5$), *Lrat*$^{-/-}$ ($n = 5$), and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ ($n = 4$) littermates 4 h after administration by gavage of a physiologic dose of retinol (6 μg of retinol containing 1×10^6 ^{3}H counts/min) in 50 μl of peanut oil. Retinol (*ROH*) and retinyl ester (*RE*) levels are normalized to plasma triglyceride (*TG*) levels. All values are given as means $\pm$ 1 S.D. The PS/LO ratio is calculated as ((moles of retinyl palmitate + moles of retinyl stearate)/(moles of retinyl linoleate + moles of retinyl oleate)). Statistical significance is as follows: *a*, $p < 0.01$ compared with the corresponding level in wild type mice; *b*, $p < 0.01$ compared with *CrbpII*$^{-/-}$ mice.

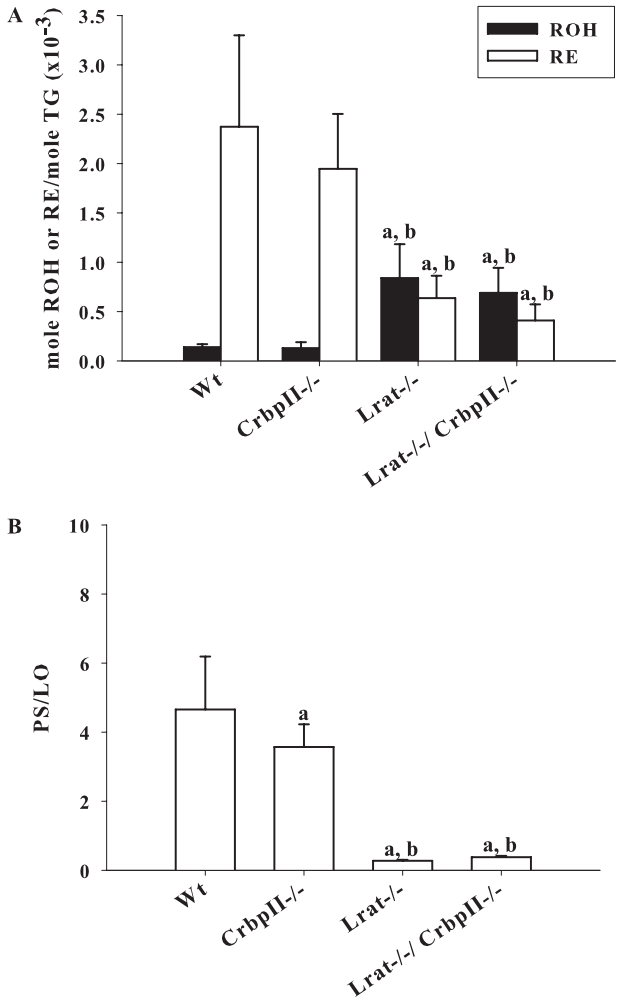

FIGURE 4. **CRBPII expression does not directly block retinyl ester formation by intestinal ARATs.** Retinol (*black bars*) and retinyl ester (*open bars*) levels (*panel A*) and PS/LO ratio (*panel B*) in postprandial serum obtained from P-407-treated male wild type (*Wt*) ($n = 12$), *CrbpII*$^{-/-}$ ($n = 23$), *Lrat*$^{-/-}$ ($n = 6$), and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ ($n = 4$) littermates 4 h after administration by gavage of a pharmacologic dose of retinol (1,000 μg of retinol) in 50 μl of peanut oil. Retinol (*ROH*) and retinyl ester (*RE*) levels are normalized to plasma triglyceride (*TG*) levels. All values are given as means $\pm$ 1 S.D. The PS/LO ratio is calculated as ((moles of retinyl palmitate + moles of retinyl stearate)/(moles of retinyl linoleate + moles of retinyl oleate)). Statistical significance is as follows: *a*, $p < 0.01$ compared with the corresponding level in wild type mice; *b*, $p < 0.01$ compared with *CrbpII*$^{-/-}$ mice.

ARAT activities in retinyl ester synthesis in response to consumption of a large dose of retinol. These data support the hypothesis that CRBPII channels newly absorbed retinol to LRAT for esterification.

To investigate whether CRBPII acts specifically to block or prevent the involvement of intestinal ARAT activities in retinyl ester synthesis from newly absorbed retinol, we generated *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ mice. The live born mutants were fertile and viable. We administered both physiological (Fig. 3) and pharmacological (Fig. 4) doses of retinol to P-407-treated age- and gender-matched wild type, *CrbpII*$^{-/-}$, *Lrat*$^{-/-}$, and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ littermates and assessed retinoid absorption in the postprandial circulations of the mice 4 h after dose administration. As can be seen in Figs. 3 and 4, the amounts of retinol and retinyl esters present in the postprandial circulations of *Lrat*$^{-/-}$ and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ mice are not statistically different. As would be expected because of the disruption of *Lrat*, for both doses of retinol, postprandial retinyl ester levels were significantly lower in the *Lrat*$^{-/-}$ and *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ mice as compared with wild type or *CrbpII*$^{-/-}$ mice, whereas postprandial levels of unesterified retinol were significantly higher for the high dose. If CRBPII directly blocks retinol from being acted upon by intestinal ARAT activities present in the intestine, one would expect to observe greater amounts of intestinal retinyl ester synthesis in *Lrat*$^{-/-}$/*CrbpII*$^{-/-}$ mice as compared with *Lrat*$^{-/-}$, which express CRBPII. This is clearly not the case. In a similar vein, for Fig. 3, *panel B*, the PS/LO ratio is not different for wild type compared with *CrbpII*$^{-/-}$ mice suggesting that the absence of CRBPII does not result in increased acyl-CoA-dependent retinyl ester formation in response to a physiologic challenge of retinol. This is not the case for a pharmacological challenge of retinol where the PS/LO ratio

observed for *CrbpII*$^{-/-}$ mice was significantly less than that of wild type mice (see Fig. 4, *panel B*). Thus, in response to a large retinol dose, acyl-CoA-dependent retinyl ester formation becomes more prominent when CRBPII is absent. Collectively we take these data to suggest that although CRBPII acts to facilitate LRAT catalysis of retinyl ester upon challenge with retinol, it does not act *in vivo* directly to block retinol availability to ARATs.

As it is well established that retinoic acid is synthesized in the intestine in response to administration of a bolus of retinol or upon consumption of a retinol-rich meal (34), we wondered whether CRBPII may act in mediating retinoic acid formation from either retinol or from retinaldehyde that might be formed upon cleavage of proretinoid carotenoids. To assess this, we prepared intestinal scrapings from the proximal 5 cm of small intestine from both *CrbpII*$^{-/-}$ and wild type mice that had been fasted overnight. Using experimental conditions originally described by others to assess retinol metabolism by rat mucosal scrapings (26), we incubated the freshly prepared mucosal scraping for 1 h at 37 °C with either retinol or retinaldehyde and then assessed retinoic acid concentrations produced from these retinoid precursors by normal phase HPLC. To ensure that the amount of retinoid added to the incubation did not exceed the availability of CRBPII present in the scrapings from wild type mice, the amount of retinol or retinaldehyde added to the incubation mixtures was calculated to constitute, on a molar basis, only 50% of the concentration of CRBPII expected to be present in the wild type mucosa scrapings. We readily detected retinoic acid formation from exogenously added retinaldehyde. However, the quantities of retinoic acid produced upon incubation in mucosal scrapings prepared from *CrbpII*$^{-/-}$ or wild type mice were not different (4.6 ± 1.4 pmol of retinoic acid formed per h/mg of protein for *CrbpII*$^{-/-}$ mice ($n = 3$) *versus* 2.7 ± 0.3 pmol/h/mg of protein for wild type mice ($n = 3$)). We were not able to detect retinoic acid formation when retinol was added to the mucosal scrapings. Thus, these *in vitro* experiments do not suggest possible actions of CRBPII in mediating retinoic acid formation from recently ingested retinol or retinaldehyde (which would be formed through cleavage of dietary β-carotene).

DISCUSSION

We have used a number of established and new mouse models to gain insight into the biochemical processes and factors that are important for mediating dietary retinoid uptake from the intestine. Our data establish that the triglyceride-synthesizing enzyme DGAT1 is an intestinal ARAT that contributes *in vivo* to retinyl ester synthesis in the enterocyte. Interestingly, although DGAT1 is highly expressed in adipose tissue, DGAT1 does not contribute significantly to retinyl ester synthesis in adipocytes. We obtained evidence that CRBPII acts *in vivo* to channel retinol to LRAT for esterification. This finding agrees fully with proposals based on *in vitro* data presented in the literature (10, 17–20). Contrary to what has been proposed in the literature based on *in vitro* evidence, *in vivo* CRBPII does not act directly to protect newly absorbed retinol from the actions of intestinal ARATs.

DGAT1 Is a Relevant ARAT in the Intestine but Not in Adipose Tissue—Although the involvement of an acyl-CoA-dependent enzyme or ARAT in the synthesis of retinyl esters was first proposed for the intestine and liver more than 2 decades ago (12–14), the identity of this enzyme has remained obscure. In 2005, three groups independently reported *in vitro* studies that demonstrated DGAT1 can catalyze the acyl-CoA-dependent esterification (11, 15, 16). This study establishes that DGAT1 acts *in vivo* as an intestinal ARAT. Ross and co-workers (12, 20, 28) proposed that ARAT involvement in retinyl ester synthesis becomes relatively more important as the amount of retinol available from the diet increases. Our findings fully support this notion. In response to a physiological dose of retinol, LRAT is the predominant enzyme catalyzing intestinal retinyl ester synthesis. We speculate that DGAT1 only acts as an intestinal ARAT when the amount of retinol being absorbed exceeds the capacity of LRAT to process this retinol. However, our data do not truly allow us to distinguish whether DGAT1 normally catalyzes a small amount of acyl-CoA-dependent retinyl ester formation, possibly up to 10% of total, in response to a physiological challenge with retinol, or whether DGAT1 only takes on the role of an ARAT in response to a physiological retinol challenge when LRAT is absent from the intestine. Our data are clear, however, that in response to a large pharmacologic dose of retinol, DGAT1 contributes very substantially toward intestinal retinyl ester synthesis, accounting for as much retinyl ester synthesis as LRAT. These data establish that the actions of DGAT1 as an intestinal ARAT become more significant in response to greater retinol availability in the intestine. Moreover, our data establish that another ARAT activity (or activities) present in the small intestine also contributes toward retinyl ester formation when the intestine is challenged with a large pharmacological dose of retinol. Thus, in addition to DGAT1, there is at least one other enzyme present in the intestine that can act as an ARAT in response to a large dose of retinol.

We (11) and Liu and Gudas (29) have reported that both retinol and retinyl ester levels are elevated 2–3-fold in gonadal and perirenal adipose tissue obtained from *Lrat*$^{-/-}$ mice compared with age-, gender-, and diet-matched wild type mice. The cellular site of retinyl esters within adipose tissue is the adipocyte (35, 36). Because DGAT1 is highly expressed in adipocytes (30), our working hypothesis had been that DGAT1 was responsible for catalyzing adipose tissue retinyl ester formation. But our data (Table 2) proved this hypothesis incorrect. Adipose tissue retinol and retinyl ester levels are not statistically different either for *Lrat*$^{-/-}$ compared with *Lrat*$^{-/-}$/*Dgat1*$^{-/-}$ mice or for wild type compared with *Dgat1*$^{-/-}$ mice. These data argue that DGAT1 does not act significantly in adipose tissue to catalyze retinyl ester synthesis and raise two important questions regarding retinyl ester synthesis in adipose tissue. The first question is as follows: what is the identity of the enzyme or enzymes that catalyze retinyl ester synthesis in adipose tissue? Our studies indicate that recombinant DGAT2, ATGL, and HSL, lipid-metabolizing enzymes that are highly expressed in adipose tissue (31, 33, 37–40), are unable to catalyze *in vitro* retinyl ester synthesis. Farese and co-workers (15, 41) have demonstrated that acyl-CoA:monoacylglycerol acyl-

transferase 1 (often referred to as MGAT1) and a multifunctional acyltransferase, termed MFAT, are able *in vitro* to catalyze retinyl ester formation from exogenous fatty acyl-CoAs and retinol. Possibly these enzymes or other similar enzymes catalyze retinyl ester synthesis in adipose tissue. There is considerable merit in resolving this question because RBP synthesized in adipose tissue is proposed to be an adipokine that signals peripheral tissue in a manner that modulates insulin responsiveness (42). The sole established action of RBP is to mobilize retinol from tissue retinoid stores (43, 44). Consequently, if one is to understand fully the role of RBP in signaling/regulating insulin responsiveness, there is a need to understand how adipose tissue retinoid stores accumulate and how this relates to adipose tissue RBP physiology. The second question raised by our data centers on how or why DGAT1 can act in the intestine as an ARAT but not have this same role in adipose tissue where it is also highly expressed (30). We cannot provide a definitive answer to this question. It is tempting to speculate that as yet unidentified differences in the biochemical properties of the cellular retinol-binding proteins may account for this difference in DGAT1 actions in the intestine compared with adipose tissue. The enterocytes express predominantly CRBPII (10, 45, 46), whereas adipocytes express CRBPIII (47) and the cells composing the stromal vascular cell fraction obtained from adipose tissue express CRBPI (35, 47). Moreover, adipose tissue levels of CRBPIII protein are elevated by ~3-fold in *Lrat*$^{-/-}$ mice (11). Perhaps CRBPIII channels retinol metabolically to a specific enzyme expressed in adipocytes that catalyzes retinyl ester formation and/or prevents DGAT1 from exerting its ARAT activity on CRBPIII-bound retinol.

What Role Does CRBPII Have in Mediating Retinol Absorption?—CRBPII is highly expressed in enterocytes and represents 0.4–1% of the total cytosolic protein (10, 27, 45, 46). In the adult, CRBPII expression is limited to the small intestine, although CRBPII is highly expressed in the embryonic liver and lung (10, 29, 45, 46). The literature, based on *in vitro* studies, has proposed that intestinal CRBPII acts metabolically to channel retinol to LRAT and to prevent retinol utilization by intestinal ARATs (10, 17–20). As far as we are aware, these hypotheses have not been tested through *in vivo* studies. Our data support the first of these hypotheses that CRBPII channels retinol to LRAT for esterification. This is evidenced in Table 3 where the PS/LO ratio of intestinally synthesized retinyl esters supports the conclusion that the absence of CRBPII results in less retinol being acted upon by LRAT. However, our data do not support the idea that CRBPII directly prevents retinol from being esterified by intestinal ARATs. If CRBPII directly prevents or limits retinol availability for ARAT action, one would expect to observe an increase in intestinal retinyl ester output for *Lrat*$^{-/-}$*/CrbpII*$^{-/-}$ mice as compared with *Lrat*$^{-/-}$ mice that express CRBPII. We did not observe this for either the physiological or pharmacologic doses of retinol (Figs. 3 and 4). Based on these findings, we conclude that *in vivo* CRBPII does not directly block retinol availability to intestinal ARATs. However, a subtle point needs to be stressed. By metabolically channeling retinol for LRAT action, this effectively diminishes retinol availability for ARAT-catalyzed retinyl ester formation, but this effect is not a direct one.

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