Increased Neonatal Mortality in Mice Lacking Cellular Retinol-binding Protein II

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Cellular retinol-binding protein II (CRBP II) is a member of the cellular retinol-binding protein family, which is expressed primarily in the small intestine. To investigate the physiological role of CRBP II, the gene encoding CRBP II was inactivated. The saturable component of intestinal retinol uptake is impaired in CRBP II−/− mice. The knockout mice, while maintained on a vitamin A-enriched diet, have reduced (40%) hepatic vitamin A stores but grow and reproduce normally. However, reducing maternal dietary vitamin A to marginal levels during the latter half of gestation results in 100% mortality/litter within 24 h after birth in the CRBP II−/− line but no mortality in the wild type line. The neonatal mortality in heterozygote offspring of CRBP II+/− dams (79 ± 21% deaths/litter) was increased as compared with the neonatal mortality in heterozygote offspring of wild type dams (29 ± 25% deaths per litter, p < 0.05). Maternal CRBP II was localized by immunostaining in the placenta at 18 days postcoitum as well as in the small intestine. These studies suggest that both fetal as well as maternal CRBP II are required to ensure adequate delivery of vitamin A to the developing fetus when dietary vitamin A is limiting.

Vitamin A or retinol is an essential nutrient that is required for growth, reproduction, fetal development, and vision (1–3). Within cells, retinol can be oxidized reversibly to retinal and subsequently irreversibly oxidized to retinoic acid. The effects of retinoic acid are directly mediated through nuclear retinoic acid receptors, which are ligand-dependent transcriptional activators belonging to the nuclear receptor family (3–5). Retinol supports all known functions of vitamin A, but retinoic acid does not support the function of vision (since retinal is the chromophore) or reproduction except when administered in pharmacological doses (1–3).

In adult animals, CRBP II is primarily expressed in high abundance in small intestinal villus absorptive cells, where it represents 0.4–1% of the total cytosolic protein (15). During embryonic days E8.5–9.5, CRBP II transcripts have been detected in uterine decidua and trophoblastic cells, and between E10.5 and E15, CRBP II transcripts are restricted to the yolk sac (16). Toward the end of gestation, CRBP II appears in the small intestine and is expressed throughout life, and CRBP II is also expressed transiently in perinatal liver and lung (17–19). In contrast, CRBP I is expressed at high levels in the liver, kidney, eye, and genital tract but at very low levels within the small intestinal mucosa (10, 11, 17, 18). Immunohistochemical localization of CRBP I in the small intestine revealed staining in the connective tissue cells in the lamina propria and in cells located within the gut-associated lymphoid tissue but not in epithelial cells (15). Additional murine and human cellular retinol-binding proteins, both named CRBP III, were recently described (12–14). Murine CRBP III, also termed Rbp7 using genetic nomenclature, is expressed in adipose tissue and muscle (12, 13). Human CRBP III is expressed in the liver and kidney, similar to CRBP I (14), and will be designated as CRBP IV in this discussion.

The in vivo roles of CRBP I have been addressed through the generation and characterization of CRBP I−/− mice (20). CRBP I−/− mice appear healthy and fertile while they are maintained on a vitamin A-enriched diet, but hepatic stores of retinyl esters are reduced by 50%. CRBP I knockout mice exhibit a shorter elimination half-life of liver retinoids and develop vitamin A deficiency more rapidly when subjected to a vitamin A-free diet (20, 21).

The tissue localization of CRBP II suggests that it is specifically adapted for intestinal absorption and metabolism of retinol. Stably transfected differentiated Caco-2 cells overexpressing CRBP II exhibit increased uptake and esterification of all-trans-retinol (22). The magnitude of retinol esterification and retinol absorption was directly related to the total CRBP II content. The interconversion of retinol and retinal is mediated by multiple microsomal and cytosolic retinol dehydrogenase activities (23, 24). The addition of CRBP II to cell extracts stimulates the microsomal reduction of retinol to retinal but inhibits cytosolic reduction of retinal (24). To directly investigate the in vivo role of CRBP II in intestinal absorption and metabolism, we have generated CRBP II null mutant mice by targeted disruption of the mouse CRBP II gene.

1 The abbreviations used are: CRBP, cellular RBP; RBP, retinol-binding protein; ARAT, acyl-CoA:retinyl acyltransferase; LRAT, lecithin:retinyl acyltransferase; E, embryonic day.
CRBP II Knockout Mice

**EXPERIMENTAL PROCEDURES**

Construction of the Targeting Vector—Genomic clones containing exon 1 and exon 2 of the mouse CRBP II gene were obtained from a 129/Sv mouse P1 library (Genome Systems). Mapping and sequencing of genomic clones revealed that the mouse CRBP II gene organization is similar to the rat homologue (25). The structure of the targeting vector in which a neomycin cassette was inserted into exon 1 is depicted in Fig. 1A.

Generation of CRBP II−/− Mice—129/Sv embryonal stem cells were electroporated with the construct and then put under positive selection with G418. One of 120 G418-resistant clones was shown to exhibit the Southern blot pattern expected for a single homologous recombination event. It was injected into C57BL/6J blastocysts to create chimeric mice, of which three males and one female transmitted the mutation to their offspring. Mice were genotyped initially by Southern blotting (see Fig. 1B). Once the mutant line was established, the polymerase chain reaction was used to genotype the animals.

Mice and Diets—Mice were initially bred in a mixed 129/Sv-C57BL6/J genetic background, and then backcrossed against a C57BL6/J background for at least six generations. The mice were maintained on a vitamin A-enriched rodent chow diet (PicoLab® Rodent Diet 20) containing 25 IU of retinyl ester/g. The mice were maintained under barrier conditions and fed irradiated diets. In certain experiments, the mice were placed on defined diets with marginal vitamin A content or sufficient vitamin A content as described previously (26). The marginal vitamin A diet was a modified AIN-95G purified vitamin A-free diet (Dyets catalog no. 119134, Bethlehem, PA) with 0.6 IU of retinyl palmitate/g (Dyets catalog no. 119135). The sufficient vitamin A diet was a modified AIN-95G purified vitamin A-free diet with 4 IU of retinyl palmitate/g. Mice were placed overnight in a mating cage. Noon of the day that a vaginal plug was observed was designated embryonic day 0.5 postcoitum. Tissues were fixed in 10% buffered formalin and embedded in paraffin. 5-μm paraffin sections were used for immunohistochemistry.

**Immunohistochemistry—**Tissues were homogenized in TRIZol reagent (Invitrogen), and total RNA was isolated according to the manufacturer’s recommendation based on improvements to the single-step RNA isolation method developed by Chomczynski and Sacchi (27). Northern blots were prepared and probed with radiolabeled cDNAs. The membranes were washed once with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for 15 min at 25 °C, once with 0.1× SSC, 0.1% SDS for 15 min at 25 °C, and twice with 0.1× SSC, 0.1% SDS for 15 min at 65 °C. The following cDNA probes were used: rat CRBP I cDNA (17), rat CRBP II cDNA (18), mouse CRBP III cDNA (12, 13), human CRBP IV cDNA (14), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (17). Under these conditions, there was no cross-reactivity between the CRBP cDNA probes. The membranes were exposed to a PhosphorImager screen overnight. Signals were scanned and quantitated on a Storm PhosphorImager (Amerham Biosciences). The signal densities were normalized with respect to the glyceraldehyde-3-phosphate dehydrogenase mRNA signal.

Western blots of cytosolic extracts were incubated with rabbit polyclonal anti-rat CRBP II antibody at a dilution of 1:5,000 and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin at a dilution of 1:10,000 (28). The blots were developed with a chemiluminescent substrate (enhanced chemiluminescence (ECL), Amerham Biosciences).

**RESULTS**

Disruption of the Mouse CRBP II Gene—To construct the targeting vector, the promoter, exon I, and a portion of intron I were replaced by a neomycin cassette (Fig. 1A). One of 120 G418-resistant clones exhibited the Southern blot pattern predicted for a single homologous recombination event. This positive clone was injected into C57BL6/J blastocysts to create chimeric mice. Three male chimeras and one female transmitted the mutation to their offspring. Mice were genotyped by Southern blotting (Fig. 1B). No CRBP II protein was detected in intestinal cell extracts or liver cell extracts prepared from 1-day-old CRBP II−/− mice (Fig. 1C). There was no immunohistochemical staining with anti-CRBP II antibody in the CRBP II−/− small intestinal epithelium (Fig. 2). Northern blot analysis revealed no detectable CRBP II mRNA in CRBP II−/− intestinal RNA (Fig. 3A). As expected, hepatic CRBP II mRNA became undetectable in adult wild type mice (Fig. 3B). These studies prove that the mouse CRBP II gene was successfully disrupted.

CRBP II Null Mutant Mice Appear Healthy and Viable on a Vitamin A-enriched Diet—When the animals were fed a vitamin A-enriched diet (25 IU/g diet), the CRBP II genotype of the offspring resulting from CRBP II−/− × CRBP II−/− matings segregated in a Mendelian ratio (26% CRBP II−/−, 51% CRBP II+/−, and 23% CRBP II+/+). There were no significant differences between the fasting serum retinoid levels of 3-month-old male CRBP II−/− mice (27 ± 4 μg/dl, n = 3), CRBP II+/− (23 ± 6 μg/dl, n = 11), and CRBP II+/+ (30 ± 2 μg/dl, n = 7) mice or between the fasting serum retinoid levels of 3-month-old female CRBP II−/− (22 ± 3 μg/dl, n = 4), CRBP II+/− (14 ± 6 μg/dl, n = 7), and CRBP II+/+ (15 ± 3 μg/dl, n = 5) mice. There was also no significant difference in the serum retinol-binding protein levels of CRBP II−/− and CRBP II+/+ mice (data not shown).

To minimize genetic variability, the disrupted CRBP II allele was backcrossed to the C57BL6/J background for six genera-
Both wild type and CRBP II null lines in the C57BL/6 background produced average litters of six pups with no significant deviation from the expected male/female ratio of 1:1. Thus, under conditions in which the animals are fed a vitamin A-enriched diet, the absence of CRBP II does not give rise to either impaired fertility or embryonic and fetal survival.

Other members of the CRBP family are expressed in the small intestine and in the liver. RNA from the small intestine and the liver was analyzed by Northern blotting to determine whether expression of other cellular retinol-binding proteins was altered in CRBP II−/− mice (Fig. 3). Low levels of CRBP I, CRBP III, and CRBP IV mRNA were detected in the small intestine of wild type and CRBP II−/− mice. Intestinal CRBP III and CRBP I mRNA levels were modestly increased by 1.5- and 2-fold, respectively, in the CRBP II−/− mice, but CRBP IV mRNA levels were not significantly different from those of wild type mice. Hepatic CRBP III mRNA was not detectable in either wild type or CRBP II−/− mice. CRBP II−/− hepatic CRBP I mRNA levels were decreased by 40%, but CRBP II−/− hepatic CRBP IV mRNA levels were increased ~2-fold as compared with wild type levels.

At low retinol concentrations, [3H]-labeled all-trans-retinol uptake in isolated jejunal segments from CRBP II−/− mice was 50% less than jejunal segments from wild type mice (n = 3, p < 0.05). When a large excess of unlabeled retinol (100 μM) was added to assess the nonsaturable component of retinol uptake, there was no difference between the CRBP II−/− and wild type intestinal uptake. In addition, compatible with data using gut sheets from suckling rats (29), ~50% of the retinol uptake represented the nonsaturable component. There was no significant difference between intestinal ARAT activity in CRBP

![Fig. 1. Disruption of the CRBP II gene. A, schematic of the mouse CRBP II locus and the targeting vector. Exon I and the PGK-Neo (neomycin) cassette are shown as solid boxes. The genomic fragments obtained after an NcoI digest are indicated for wild type and homologous recombinant alleles. B, Southern blot (NcoI digest) of DNA from offspring of a CRBP II−/− intercross that was probed with 3′ external probe A. C, Western blot analysis of 300 μg liver (L) and small intestinal (I) cytosolic extracts from wild type (+/+), heterozygous (+/−), and homozygous (−/−) neonatal littermates.](http://www.jbc.org/)

![Fig. 2. Immunohistochemical localization of CRBP II in the small intestinal mucosa of 3-month-old CRBP II−/−, CRBP II−/−, and CRBP II−/− mice. CRBP II expression is restricted to villus epithelial cells in the small intestine. CRBP II expression is reduced in CRBP II−/− mice and is undetectable in CRBP II−/− mice. Magnification is ×400.](http://www.jbc.org/)

![CRBP II Knockout Mice](http://www.jbc.org/)
CRBP II Knockout Mice

Comparison of CRBP II<sup>−/−</sup> and wild-type liver retinoid levels

|          | Total ROH | Free ROH |
|----------|-----------|----------|
| CRBP II<sup>−/−</sup> | 480 ± 50 (n = 3)<sup>*</sup> | 3 ± 2 (n = 3) |
| CRBP II<sup>+/−</sup> | 830 ± 60 (n = 3)<sup>*</sup> | 15 ± 15 (n = 3) |

<sup>*</sup> p < 0.05.

Fig. 3. Altered expression of cellular retinol-binding proteins in CRBP II<sup>−/−</sup> mice. Total cellular RNA was prepared from the proximal small intestine and the liver of 3-month-old mice consuming a vitamin A-enriched diet. A, the relative concentrations of CRBP I, CRBP II, CRBP III, and CRBP IV mRNA in CRBP II<sup>−/−</sup> and CRBP II<sup>+/−</sup> small intestines calculated from densitometric measurements of the signal intensities of the CRBPs relative to that of glyceraldehyde-3-phosphate dehydrogenase (see "Experimental Procedures"). B, the relative concentrations of CRBP I, CRBP II, CRBP III, and CRBP IV mRNA in CRBP II<sup>−/−</sup> and CRBP II<sup>+/−</sup> livers. The asterisk (*) indicates a significant difference (p < 0.05) between CRBP II<sup>−/−</sup> and CRBP II<sup>+/−</sup> levels.

<sup>II</sup> mice (180 ± 40 pmol/min/mg, n = 3) and CRBP II<sup>+/−</sup> (200 ± 40 pmol/min/mg, n = 3). There was also no significant difference between the intestinal lecithin:retinyl acyltransferase activity in CRBP II<sup>−/−</sup> mice (200 ± 20 pmol/min/mg, n = 3) and CRBP II<sup>+/−</sup> mice (200 ± 30 pmol/min/mg, n = 3).

As shown in Table I, total hepatic retinol content (retinol and retinyl esters) was reduced by 40% in CRBP II<sup>−/−</sup> mice. This was largely due to differences in retinyl esters since less than 5% of the total retinol content was unesterified retinol. No significant difference was detected between CRBP II<sup>−/−</sup> hepatic LRAT (80 ± 20 pmol/min/mg) and CRBP II<sup>+/−</sup> hepatic LRAT (90 ± 20 pmol/min/mg, n = 3) activity or between CRBP II<sup>−/−</sup> hepatic ARAT (100 ± 20 pmol/min/mg, n = 3) and CRBP II<sup>+/−</sup> hepatic ARAT (100 ± 20 pmol/min/mg, n = 3) activity.

Dietary Vitamin A Restriction Results in High Neonatal Mortality in CRBP II<sup>−/−</sup> Mice—When CRBP II<sup>−/−</sup> mice are fed a vitamin A-enriched rodent chow diet (25 IU/g), they appear to absorb enough vitamin A to support all vitamin A-dependent processes normally, although hepatic stores of vitamin A are reduced. Since intestinal retinol uptake is impaired at low retinol concentrations in CRBP II<sup>−/−</sup> mice, we reasoned that under conditions of low dietary intake, CRBP II<sup>−/−</sup> mice may not be able to absorb sufficient vitamin A. To address this question, pregnant dams were placed on marginal (0.6 IU/g) or sufficient (4 IU/g) vitamin A diets beginning on day 10 of gestation. This marginal vitamin A diet limited vitamin A intake by the dams and by the offspring during the postwean-

ing period and was originally designed to produce pups with reduced hepatic retinol content and adequate serum levels without interfering with the ability of dams to produce healthy viable offspring. When CRBP II<sup>−/−</sup> dams mated with CRBP II<sup>+/−</sup> males were fed the marginal vitamin A diet from day 10 of gestation onwards, all of the pups died within 24 h of birth (see Table II). In contrast, no deaths were observed in five litters resulting from matings between wild type females and males when the female dams were subjected to the same protocol.

To determine whether the neonatal lethality observed on the marginal diet was due to maternal or fetal CRBP II deficiency, the neonatal mortality of heterozygote pups born to CRBP II<sup>−/−</sup> dams and to wild type dams was compared. As shown in Table II, there was increased neonatal mortality in heterozygote offspring of CRBP II<sup>−/−</sup> dams (27 ± 21% deaths/litter) as compared with the heterozygote offspring of wild type dams (27 ± 26%, p < 0.05). The mortality observed in the heterozygote pups was increased as compared with that of the wild type pups of wild type dams (p < 0.05) subjected to a marginal vitamin A diet during the latter half of gestation, indicating that reduction of fetal CRBP II is also detrimental. There was no difference in food intake between the various experimental groups. There was no difference in the average litter size (six pups) between the different experimental groups.

Heterozygote pups born to CRBP II<sup>−/−</sup> dams that were subjected to a marginal vitamin A diet on day 10 of gestation appeared cyanotic and less active at birth. The most striking abnormality on gross examination was an enlarged right atrium (see Table II). In contrast, no deaths were observed in five heterozygous offspring of wild type dams mated with CRBP II<sup>−/−</sup> males. Microscopic inspection of the right atrium and right ventricle revealed right-sided but not left-sided chamber enlargement. The right atrial and right ventricular muscle appeared, however, to be of normal thickness. In contrast the hearts in neonatal heterozygous offspring from wild type, dams fed the same marginal vitamin A diet appeared normal (Fig. 4B). Although the lungs appeared grossly normal at birth, microscopic examination showed hemorrhage and decreased air space in the lungs of heterozygous offspring of CRBP II<sup>−/−</sup> dams (Fig. 4C) as compared with those of wild type dams (Fig. 4D). Immunohistochemical staining with an antibody directed against surfactant B showed decreased staining in the distal lung air spaces of the heterozygote pups born to a CRBP II<sup>−/−</sup> dams fed a marginal vitamin A diet (Fig. 4, E and F). We observed none of the gross skeletal, craniofacial, cardiovascular, or urological deformities described previously in vitamin A deficiency.

Fetal lung and liver retinol and retinyl ester levels were measured in E18 CRBP II<sup>−/−</sup> fetuses and wild type fetuses. As shown in Table III, there was no significant difference in the hepatic retinol or retinyl ester levels. The level of unesterified retinol in the pooled lungs of E18 CRBP II<sup>−/−</sup> fetuses of CRBP II<sup>−/−</sup> dams was reduced by 40% as compared with the level in the wild type fetuses of wild type dams.
of gestation, 8 days after the animals were switched to a marginal vitamin A diet (0.6 IU/g), the maternal serum retinol levels in CRBP II<sup>0/-</sup> dams (6.1 ± 0.3, n = 3) and wild type dams (6.1 ± 0.3, n = 4) were decreased by ~70% as compared with preconception serum levels (p < 0.05). Immediately after delivery, maternal serum retinol levels rebounded to preconception levels. Consistent with previous observations (16, 35), serum retinol levels did not decrease in either nonpregnant CRBP II<sup>0/-</sup> or wild type dams until after hepatic vitamin A stores were virtually exhausted (data not shown). The postpartum hepatic total retinol levels in wild type and mutant dams were 390 ± 60 μg/g (n = 4) and 250 ± 40 μg/g (n = 5, p < 0.02), respectively. Although these levels were decreased as compared with preconceptional levels, the maternal hepatic vitamin A stores were far from depleted. As opposed to the nonpregnant state, serum retinol levels decreased in both wild type and CRBP II<sup>0/-</sup> dams during gestation prior to the depletion of maternal hepatic vitamin A stores.

**CRBP II Protein Is Present in the Maternal Placenta**—To determine whether CRBP II is also expressed in the maternal placenta, immunohistochemical staining using a monospecific affinity-purified antibody directed against rat CRBP II was performed on sections of postcoitum day 18 placentas (Fig. 6). Sections of placenta corresponding to a wild type fetus and wild type mother demonstrated staining of the yolk sac (fetal origin) as well as staining in the decidua basalis and endometrial layer (maternal origin). Sections of placenta corresponding to a CRBP II<sup>0/-</sup> fetus and a CRBP II<sup>0/-</sup> null mutant mother demonstrated staining only in the yolk sac (fetal origin).

**DISCUSSION**

**CRBP II** is primarily expressed in the villus absorptive cells of the proximal small intestine. Therefore, our analysis focused initially on the effect of lacking CRBP II on intestinal absorption of retinol. Intestinal retinol absorption occurs by a saturable process at luminal retinol concentrations less than 300 nM and by a nonsaturable process (passive diffusion) at concentrations exceeding 400 nM (35–39). Only the saturable uptake of retinol is impaired in CRBP II<sup>0/-</sup> mice.

Within the enterocyte, retinol is primarily esterified with long chain fatty acids and packaged into chylomicrons. Approximately 70% of the chylomicron-associated retinyl esters is taken up by the liver, which is the major repository for vitamin A stores in the body. The liver secretes retinol complexed with serum retinol-binding protein RBP into the circulation for delivery to target tissues. Serum retinol-RBP levels are maintained at constant levels except at extremes of vitamin A nutrition. CRBP II<sup>0/-</sup> mice are able to maintain normal serum retinol levels despite hepatic stores of total retinol (free and esterified retinol) that are reduced by 40%. Serum retinol levels are normal, but hepatic stores of total retinol (free and esterified retinol) are reduced by 40% in CRBP II<sup>0/-</sup> mice. Reduced hepatic stores of total retinol have also been observed in CRBP II<sup>0/-</sup> mice (20, 21). It is possible that reduced hepatic expression of CRBP I could play a role in diverting hepatic retinol from esterification and storage pools in order to maintain serum retinol-RBP levels.

The CRBP II<sup>0/-</sup> mice, while maintained on a vitamin A-enriched diet (25 IU/g), appeared healthy and were capable of producing viable offspring. However, when dietary vitamin A was reduced to marginal levels (0.6 IU/g) for only 10 days during the latter half of gestation, we observed a striking increase in neonatal mortality in the offspring of CRBP II<sup>0/-</sup> dams as compared with that of wild type dams. When dietary vitamin A was increased to sufficient levels (4 IU/g), neonatal mortality was greatly reduced in the heterozygote offspring of CRBP II<sup>0/-</sup> dams, suggesting that the increased neonatal mor-

**TABLE II**

| Paternal genotype | Maternal genotype | Sufficient vitamin A diet (4 IU/g) % death/litter ± S.D. | Marginal vitamin A diet (0.6 IU/g) % death/litter ± S.D. |
|-------------------|-------------------|------------------------------------------------------|------------------------------------------------------|
| −/−               | −/−               | ND                                                   | 100 ± 0% (n = 2)                                     |
| +/+               | −/−               | 6 ± 7% (n = 4)                                       | 79 ± 21% (n = 5)                                     |
| −/−               | +/+               | 0 ± 0% (n = 4)                                       | 27 ± 25% (n = 5)                                     |
| +/+               | +/+               | 0 ± 0% (n = 2)                                       | 0 ± 0% (n = 5)                                       |

**FIG. 4.** Comparison of heart and lung morphology of a newborn CRBP II<sup>0/-</sup> pup of CRBP II<sup>0/-</sup> dams (left) and CRBP II<sup>0/+</sup> dams (right) fed a marginal vitamin A diet. A and B, section of the right atrium (RA) of a newborn CRBP II<sup>0/-</sup> pup born of a CRBP II<sup>0/-</sup> dam stained with hematoxylin and eosin. Magnification is ×40. C and D, section of the lung of a neonatal heterozygous pup born of a CRBP II<sup>0/-</sup> dam stained with hematoxylin and eosin. Magnification is ×100. E and F, immunohistochemical staining for surfactant B (brown) in a section of the lung of a newborn CRBP II<sup>0/-</sup> pup born of a CRBP II<sup>0/-</sup> dam counterstained with hematoxylin. Magnification is ×100.

**Maternal Serum Retinol Levels and Hepatic Total Retinol Decrease in CRBP II<sup>0/-</sup> and Wild Type Dams during Pregnancy**—Maternal serum retinol levels were measured in the mice that were switched to a marginal vitamin A diet on day 10 of gestation. As shown in Fig. 5, prior to mating, there were no differences in the serum retinol levels of CRBP II<sup>0/-</sup> and wild type female mice. On day 10 of gestation, before the mice were switched from the vitamin A-enriched diet (25 IU/g), maternal serum retinol levels decreased 50–70% in both CRBP II<sup>0/-</sup> dams (5.4 ± 0.9, n = 4) and wild type dams (9.6 ± 1.3 μg/dl, n = 3) as compared with preconception levels (p < 0.05). On day 18 of gestation, 8 days after the animals were switched to a marginal vitamin A diet (0.6 IU/g), the maternal serum retinol levels in CRBP II<sup>0/-</sup> dams (6.1 ± 0.3, n = 3) and wild type dams (6.1 ± 0.3, n = 4) were decreased by ~70% as compared with preconception serum levels (p < 0.05). Immediately after delivery, maternal serum retinol levels rebounded to preconception levels. Consistent with previous observations (16, 35), serum retinol levels did not decrease in either nonpregnant CRBP II<sup>0/-</sup> or wild type dams until after hepatic vitamin A stores were virtually exhausted (data not shown). The postpartum hepatic total retinol levels in wild type and mutant dams were 390 ± 60 μg/g (n = 4) and 250 ± 40 μg/g (n = 5, p < 0.02), respectively. Although these levels were decreased as compared with preconceptional levels, the maternal hepatic vitamin A stores were far from depleted. As opposed to the nonpregnant state, serum retinol levels decreased in both wild type and CRBP II<sup>0/-</sup> dams during gestation prior to the depletion of maternal hepatic vitamin A stores.

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a wild type mother. Magnification is
from a postcoitum day 18 conceptus derived from a wild type fetus and
3
3
CRBP II
CRBP II
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Table III
Fetal liver and lung retinoid levels
E18 CRBP II−/− and wild type fetuses were harvested by hysterotomy from CRBP II−/− and wild type dams, fed a marginal vitamin A diet,
respectively. The mean level of unesterified retinol (ROH) and retinyl esters (RE) ± S.D. are shown.

|        | Liver |        |        | Lung |        |
|--------|-------|--------|--------|------|--------|
|        | µg/g  | RE     | µg/Vg  | RE   | µg/Vg  |
| CRBP II−/− | 0.20 ± 0.04 (n = 6) | 4.6 ± 1.2 (n = 6) | 0.035 ± 0.01 (n = 15)* | 0.30 ± .03 (n = 15) |
| CRBP II+/+ | 0.24 ± 0.04 (n = 6) | 4.6 ± 1 (n = 6) | 0.054 ± 0.01 (n = 12)* | 0.049 ± .3 (n = 12) |

* p < 0.05.

Fig. 5. Serum retinol levels in female CRBP II−/− and wild type mice during pregnancy. Timed pregnant female mice (n = 3–4) were
ed a vitamin A-enriched diet (25 IU/g) for 10 days and switched to a
defined marginal vitamin A diet (0.6 IU/g). The serum retinol levels
were measured just prior to mating, on day 10 and day 18 of gestation,
and on postpartum day 1, as described under “Experimental Procedures.”

Colocolical doses of all-trans-retinoic acid; however, the offspring
die shortly after birth (3, 8, 9, 40). The neonatal deaths ob-
erved in the offspring of Vitamin A-deficient dams supple-
mented with the lower dose (12 µg/g diet) of retinoic acid and a
single 2-µg dose of retinol at day 10 of gestation appeared to be
due to delayed perinatal lung maturation (40). In other models
of partially vitamin A-deficient rats, altered mRNA expression
of the elastin and gas6 genes was observed in the fetal lung
(41), as were a decrease in neonatal lung weight relative to
body weight (42) and decreased surfactant A and B mRNA and
protein levels (43). Retinoic acid regulates expression of sur-
factant B, which is critical for respiratory adaptation after
birth (44). In preterm human neonates, vitamin A deficiency
is associated with an increased risk of developing respiratory
distress due to bronchopulmonary dysplasia, which may be
ameliorated by treating the infants with high dose parenteral
vitamin A (45).

Our initial analysis of the neonatal offspring of the CRBP
II−/− dams fed a marginal vitamin A diet during the latter half
of gestation detected no gross malformations. Hypoxia-induced
pulmonary vasoconstriction was associated with the develop-
ment of pulmonary hypertension and increased right heart
pressures (46). The findings of right-sided but not left-sided
chamber enlargement were consistent with respiratory insuf-
ficiency in the neonatal mice born to CRBP II−/− mice. Al-
though no differences in lung weights were observed,2 the
increased pulmonary congestion suggested injury to the lungs.
Further studies are underway to further characterize perinatal
lung development in these animals.

Increased neonatal mortality in the CRBP II−/− dams that
were fed a marginal vitamin A diet was initially unexpected
because of the prevailing concept that hepatic secretion of
retinol bound to RBP was the major mechanism of delivering
vitamin A to target tissues (47–50). However, no effect on fetal
development was observed in RBP−/− mice fed a vitamin A-en-
riched diet (51) or when vitamin A was withdrawn from the diet
during gestation.3 In addition, decreases in serum maternal
retinol levels during pregnancy in the absence of liver store
depletion have also been reported in other animals and in
humans (53–55). These changes indicate that vitamin A home-
ostasis is altered during pregnancy. The decrease in maternal
serum retinol levels toward the end of gestation could result
from increased vitamin A requirements made by the develop-
ing fetus and limitations in mobilizing maternal hepatic stores.
Direct delivery of chylomicron-associated retinyl esters to the
placenta could potentially serve as an alternative mechanism
for supplying the developing fetus. This could be facilitated by
the observed increase in maternal small intestinal CRBP II
expression that occurs toward the end of gestation (17, 52).

Both loss of maternal and loss of fetal CRBP II contribute to
increased neonatal mortality. Although maternal CRBP II is
expressed primarily in small intestinal villus absorptive cells,

2  E. Li, unpublished observations.
3  W. S. Blaner, unpublished observations.
we have also found that maternal CRBP II persists in the placenta throughout gestation. Fetal CRBP II is expressed transiently in the yolk sac, lung, and liver during development in addition to being expressed in the proximal small intestine (16–19). Because CRBP II is expressed primarily in the small intestine, we had hypothesized that its primary role was to facilitate intestinal absorption and metabolism of retinol. However, the increased neonatal mortality observed in mice lacking CRBP II may be primarily due to the loss of extraintestinal CRBP II. The distinct ligand-binding interactions and distinct tissue distributions of the four CRBPs described thus far suggest that each CRBP serves a distinct physiological function. Our initial characterization of mice lacking CRBP II suggests that CRBP II play a distinct role in ensuring adequate transport of vitamin A to the developing fetus, particularly when maternal vitamin A is limited.

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