Background: Retinoic acid receptor (RAR) activation induces cell differentiation and may antagonize cancer progression. Cellular retinol-binding protein I (CRBP-I) functions in retinol storage and its expression is lower in human cancers than in normal cells. We hypothesized that retinol storage might be linked to RAR activation and thus that lowered CRBP-I function might impair RAR activity and cell differentiation. Methods: Sarcoma virus 40-immortalized human mammary epithelial cells (MTSV1-7) devoid of CRBP-I were transfected with wild-type CRBP-I or CRBP-I point mutants with low RA binding affinity. The subcellular localization of CRBP-I was investigated in these cells and in wild-type or CRBP-I null mouse mammary epithelial cells (MECs), using indirect immunofluorescence and sucrose gradient fractionation. RAR activity was assessed using reporter gene assays. Acinar differentiation and in vivo tumor growth were assessed in reconstituted basement membrane and athymic mice, respectively. Results: In cells expressing wild-type CRBP-I but not the CRBP-I mutants, CRBP-I was found mainly in lipid droplets, the retinol storage organelle, and this localization was associated with promotion of retinol storage by wild-type CRBP-I only. RAR activity was higher and acinar differentiation was observed in cells expressing wild-type but not mutant CRBP-I. RAR antagonist treatment blocked and chronic RA treatment mimicked the CRBP-I induction of cell differentiation. Finally, CRBP-I suppressed tumorigenicity in athymic mice. Conclusions: Physiologic RAR activation is dependent on CRBP-I-mediated retinol storage, and CRBP-I downregulation chronically compromises RAR activity, leading to loss of cell differentiation and tumor progression. [J Natl Cancer Inst 2005;97:21–9]
it to retinoic acid to activate RXR-RAR (4). The results of these functional studies prompted the realization that vitamin A bioactivity in cancer may be compromised at the level of retinol metabolism. Retinol metabolism is thought to be regulated by high-affinity retinol-binding proteins, of which cellular retinol-binding protein-I (CRBP-I) was the first identified (5). The amino acid sequence of CRBP-I is highly evolutionarily conserved, and CRBP-I is expressed by many animal species (5). CRBP-I has been conceptualized as a chaperone that presents retinol to its metabolizing enzymes and was reported to be cytosolic (5).

The study of CRBP-I null mice demonstrated an essential role for this gene in central (i.e., liver) vitamin A storage, which occurs in the form of retinyl esters in organelles known as lipid droplets (6). This role was assigned in part because of the ability of CRBP-I to increase the efficiency of retinol esterification by lecithin retinyl acyltransferase (LRAT) (6). CRBP-I exerts a similar enhancement of vitamin A storage in other cell types, e.g., breast epithelial cells (7), suggesting that its retinol storage function is important in organs other than the liver. Our finding that CRBP-I is uniformly expressed in the normal breast epithelium but down-regulated in approximately 24% of human breast cancers (8), provided preliminary evidence for the concept that vitamin A bioactivity may be compromised in cancer at the level of retinol metabolism. Other studies extended support for this concept by showing that CRBP-I silencing is a common epigenetic event in many human cancers (9–11). We have reported that CRBP-I inhibits the anchorage-independent growth of sarcoma virus 40 (SV40)-transformed human breast epithelial cells (7). However, whether reduced CRBP-I function compromised local vitamin A bioactivity and whether this reduction in function led to loss of differentiation and tumor progression remained open questions. This work addresses this gap by examining the specific subcellular localization of CRBP-I and the effect of wild-type (WT) and mutant CRBP-I forms on RAR activity, cell differentiation in reconstituted basement membrane, and in vivo tumorigenicity of human and mouse breast cancer cell models.

**METHODS**

**Cell Culture**

MTSV1-7 is an SV40-immortalized human mammary epithelial cell (MEC) line that acquired anchorage-independent growth in association with endogenous CRBP-I silencing (7). WT and CRBP-I knockout mouse colonies were embryonically rederived with the assistance of Mount Sinai School of Medicine’s Mouse Genetics Shared Research Facility and housed in a barrier facility. All mouse protocols followed guidelines approved by Mount Sinai’s Institutional and Animal Care Use Committee. Mammary epithelial cells from female CRBP-I knockout mice were generated by collagenase digestion and depleted of fibroblasts by differential adhesion. Mammary epithelial cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) containing antibiotics, 5% heat-inactivated fetal bovine serum (FBS), 5 μg/mL bovine insulin, 100 ng/mL cholera toxin, 15 ng/mL epidermal growth factor, and 0.5 μg/mL hydrocortisone. Immortalized mammary epithelial cells were derived by infection with pBabePuro-SV40 T antigen as described (12), and their epithelial nature was confirmed by cytokeratin staining (positive for keratin 18 and virtually negative for keratin 14). Transfection MTSV1-7 cell pools stably transfected with empty vector (MTSVvect°) or CRBP-I (MTS-VCRBP-I) have been described (7). Isogenic MTSV1-7 cells expressing WT CRBP-I, leucine to alanine change at amino acid (aa) 29 (CRBP-I-L29A), or arginine to glutamate change at aa 58 (CRBP-I-R58E), both with lower affinity to retinol than WT, or a chimeric protein containing the aa sequence GKPIPNNPL-GLDST at the C terminus (CRBP-I-V5) were generated using the Flp-In system (Invitrogen, Carlsbad, CA). Full-length CRBP-I cDNA was subcloned into a vector containing a flip recombinase target (FRT) site, pcDNAS5/FRT, and the QuikChange Site–Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate the CRBP-I-L29A and CRBP-I-R58E mutants. CRBP-I-V5 was made by ligating polymerase chain reaction–amplified CRBP-I into pcDNAS5/FRT/V5-His TOPO TA (Invitrogen). All plasmids were confirmed by sequencing. The retinoic acid response element–enhanced green fluorescent protein plasmid (RARE-EGFP) (13) was cotransfected with pSV2/neo (BD Biosciences Clontech, Palo Alto, CA) at a 2 μg:0.2 μg ratio, and EGFP fluorescence was evaluated 24–96 hours later or after selection in G418. Luciferase reporter assays were carried out using the dual luciferase reporter system according to the manufacturer’s instructions (Promega, Madison, WI). Cells were also transfected with a plasmid containing Renilla luciferase driven by an SV40 enhancer (pRLSV40) as a control for transfection efficiency (Promega).

**Lipid Droplets**

Lipid droplet and cytosol fractions were resolved using two methods (14,15) that gave similar results in this study. To visualize lipid droplets, cells were treated with 5 μg/mL 4,4-difluoro-1,5,5,7,8-pentamethyl-4-bora 3a, 4a-diaza-s-indacene (BODIPY 493/503, Molecular Probes, Eugene, OR) or 200 nM Nile Red (Sigma, St. Louis, MO) for 16 or 2 hours, respectively, and washed with DMEM followed by phosphate buffered saline (PBS).

**Immunoblot Analysis**

CRBP-I immunoblotting was described previously (7). Briefly, cells were homogenized in hypotonic buffer (10 mM Tris pH 7.4, 1 mM EDTA pH 8.0, 7 mM 2-mercaptoethanol, supplemented with protease and phosphatase inhibitors) and centrifuged at 120 000g for 35 minutes at 4 °C in a benchtop Beckman ultracentrifuge. For other applications (SV4DT/t antigen immunoblot, standard RIPA (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mg/mL aprotinin; 1 mg/mL leupeptin; 1 mg/mL pepstatin; 1 mM Na3VO4; 1 mM NaF) lysates were prepared. Briefly, cell monolayers or cell pellets were washed once with ice-cold PBS with phosphatase inhibitors and then lysed on ice or in a rocker at 4 °C for 20 minutes, centrifuged at 14 000g for 15 minutes at 4°C to separate out the insoluble fraction, and supernatants were then recovered. Protein was assayed using the BioRad (Hercules, CA) protein assay, and 20–50 μg protein per lane were separated by electrophoresis through 10% or 12% sodium dodecyl sulfate–polyacrylamide electrophoresis gels and electrophoretically transferred onto polyvinylidene fluoride membranes. The antibodies used for immunoblotting were rabbit polyclonal anti-CRBP-I peptide (7),
mouse monoclonal anti-SV40 large T antigen/small t antigen (BD Pharmingen), and mouse monoclonal anti-beta 1 integrin (NeoMarkers, Freemont, CA). Bands were visualized by chemiluminescence using Amersham’s detection system (Amer-
sham Biosciences, Piscataway, NJ).

**Lactic Acid Dehydrogenase Assay**

Briefly, 10 μL of total lysate or lipid droplet fraction were added to 990 μL of a reaction mixture containing 0.22 mM NADH and 1.1 mM Na pyruvate in 200 mM Tris-HCl pH 7.3 at 37 °C. The formation of lactic acid from pyruvate was accom-
panied by the oxidation of NADH measured at 340 nm; the rate of loss of absorption units at 340 nm (expressed per minute) was divided by 6.2 to yield the number of micromoles of NADH converted per milliliter per minute (equal to one lactic acid hydrogenase unit).

**Retinol Autofluorescence**

MTSV cells were incubated for 24 hours in DMEM, incubated overnight at 37 °C in DMEM supplemented with 1 μM retinol and 1 mg/mL bovine serum albumin, and washed three times with PBS. Retinol autofluorescence was recorded as de-
scribed (16,17).

**Indirect Immunofluorescence**

MTSV and MECSV cells were plated at 1.5 × 10^4 cells per well and grown on coverslips (placed at the bottom of the well) overnight, fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with affinity-purified rabbit polyclonal anti-rat CRBP-I (18), mouse monoclonal anti-V5 (Invitrogen), or mouse monoclonal anti-E-cadherin (Zymed Laboratories, San Francisco, CA). Secondary goat anti-mouse or rabbit antibodies conjugated to ALEXA 488 or 568 fluorescent dyes (Molecular Probes) were used for detection. Coverslips were mounted in ProLong Antifade solution (Molecular Probes).

**Differentiation Assay**

Cells (5 × 10^3 to 1.5 × 10^4) in 500 μL DMEM with 2%-5% FBS supplemented with Matrigel (BD Biosciences, San Jose, CA) were plated in four-well plates that had been precoated with a thin layer of Matrigel. The cells were then overlaid with 250 μL of DMEM 2%-5% FBS. This medium was replaced either every 2–3 days or daily, during drug treatments. Outgrowth morphology was monitored by inverted phase contrast microscopy or hematoxylin and eosin staining. Results shown are representative of the majority (70%-90%) of the colonies.

**Microscopy**

Standard epifluorescence from immunofluorescence and retinol autofluorescence was captured with a Nikon E-700 micro-
scope using Plan-APOCHROMAT 40×, 63×, and 100× lenses through a Diagnostic Instruments SPOT digital camera (Diag-
nostic Instruments, Sterling Heights, MI). Nile Red and EGFP fluorescence were detected using an inverted Nikon Eclipse TS 100-F microscope and photographed with a Nikon COOLPIX 990 digital camera (Nikon, Melville, NY). Fluorescence background was subtracted using Adobe Photoshop 5.0 software (Adobe, San Jose, CA). Confocal microscopy and transmission electron microscopy were performed with the assistance of Mount Sinai School of Medicine’s Microscopy Shared Resource Facility.

## Tumorigenicity Assay

Xenograft experiments were done as described previously (19), using 8-week-old female athymic mice (National Cancer Institute, Frederick, MD) housed in a barrier facility in Mount Sinai’s East Building. Tumor length (L) and width (W) were measured with a vernier caliper, and tumor volume was calcu-
lated as \( \frac{L \times W^2}{2} \). All mouse protocols followed guidelines approved by Mount Sinai’s Institutional Animal Care and Use Committee.

**Statistical Analysis**

The two-sided Mann–Whitney U test was applied to the tumor volume measurements to determine statistical significance of the differences between experimental groups; \( P \) values less than .05 were considered statistically significant, and all \( P \) values are two-sided.

## RESULTS

**CRBP-I Cellular Localization**

We used WT and CRBP-I null mice and affinity-purified antibodies to investigate CRBP-I’s subcellular location. Mam-
mary epithelial cells isolated from WT and CRBP-I knockout female mice and immortalized with the SV40 T antigen (MECS-
V^WT and MECSV^Ko, respectively) were used in immunofluores-
ence assays. Contrary to the previous notion that CRBP-I is cytosolic, we found that CRBP-I in MECSV^WT cells was local-
ized primarily to perinuclear vesicles, reminiscent of lipid dro-
plets (Fig. 1A). No staining was detected in MECSV^Ko cells, confirming the specificity of the antibody (Fig. 1A). To test the no-
tion that CRBP-I localizes to lipid droplets, total lysates were prepared, aliquots were saved, and purified lipid droplet and cytosol fractions were obtained by discontinuous sucrose density ultracentrifugation (14,15). Side-by-side immunoblot analysis of total lysates and purified lipid droplets demonstrated that CRBP-I is greatly enriched in the buoyant lipid droplet fraction relative to total lysate (Fig. 1B). In five independent experi-
ments, the lipid droplet fraction accounted for 2%-2.5% of total cell protein such that if all CRBP-I protein localized to lipid droplets, an enrichment factor of 40–50-fold would be expected. The actual enrichment factor was 25–30-fold (bar graph, Fig. 2C), indicating that most of the CRBP-I protein (range of 25/50 × 100% = 50% to 30/40 × 100% = 75%) localizes to lipid droplets. Control experiments showed that the lipid droplet fraction was free of plasma membrane and cytosol contamination (beta 1 integrin immunoblot and lactic acid dehydrogenase assays, respectively; Fig. 1B, middle panel and bar graph), and immunoblot analysis of cytosol fractions confirmed that they contained little if any CRBP-I (not shown). Routine differential centrifugation did not resolve lipid droplets from the cytosol; this probably accounts for the earlier impression that CRBP-I was cytosolic. To further study CRBP-I localization, we ectopically expressed CRBP-I fused to a C-terminal V5 epitope (CRBP-I-V5) and stained cells with mouse monoclonal anti-V5 antibody and BODIPY, a fluorescent lipid droplet marker. As shown in Fig. 1C, lipid droplets were positive for V5 staining (some granular V5 staining not corresponding to lipid droplets was also seen).
CRBP-I Function in Retinol Storage and RAR Activation

A critical open question was whether CRBP-I’s regulation of breast epithelial retinol storage in turn regulates RAR activity. We hypothesized that cells might rely on locally stored retinol for generating RA and activating RARs; this hypothesis predicted that in the absence of exogenous RA, RARs would be poorly active in cells devoid of CRBP-I, such as MTSV1-7 cells. To test this hypothesis, we first derived isogenic MTSV1-7 clones expressing no CRBP-I or equal protein levels of ectopic WT CRBP-I or the CRBP-I point mutants L29A or R58E (Fig. 2A), which were previously shown to bind retinol with 20% of the affinity of WT CRBP-I (20). We then evaluated the retinol storage ability of the MTSV1-7 clone cells by monitoring retinol autofluorescence. When excited with ultraviolet light (330-380 nm), retinol emits a highly characteristic green autofluorescence that fades away within 20 seconds; retinol autofluorescence highlights the lipid droplets by virtue of their high retinyl ester content (16,17). We then evaluated RAR activity by monitoring RARE-EGFP reporter activity, which is proportional to RAR activity (13). We expected that only cells expressing WT CRBP-I would efficiently take up and store retinol from serum, and the autofluorescence data confirmed this expectation (Fig. 2B, left panels). In support of our hypothesis that decreased retinol storage might lead to decreased RAR activity, cells that were deficient in retinol storage also proved deficient in RARE-EGFP activation (Fig. 2B, cf. right and left panels). The point mutants did not concentrate in lipid droplets to the same extent as WT CRBP-I (compare lipid droplet/total lysate ratios, Fig. 2C). Transient transfection experiments confirmed that CRBP-I expression was associated with increased RARE activity and showed that CRBP-I had no effect on the activity of a RXR or peroxisome proliferator-activated receptor response element (Fig. 2D). Activating protein 1 reporter activity is transrepressed by liganded RAR (21), and its activity was lower in cells expressing CRBP-I than in those without CRBP-I expression.

Taken together, these results strongly suggest that CRBP-I promotes RAR activity by promoting increased retinol storage and utilization. In the presence of CRBP-I, retinol esterification is catalyzed by lecithin retinol acyltransferase, with a minimal contribution from acyl-CoA:retinol acyltransferase (5). Therefore, to further test the link between retinol storage and RAR activity, we overexpressed LRAT C161A, a catalytically inac-
tive lecithin retinol acyltransferase mutant (22), with the expectation that this would have a downstream inhibitory effect on RAR. Indeed, RARE-EGFP activity was lower in cells overexpressing LRAT C161A than in those not expressing it (Fig. 2E).

CRBP-I Function in Epithelial Differentiation

Given the link between CRBP-I and RAR activity, the chronic somatic loss of CRBP-I might allow tumor cells to bypass RAR-induced differentiation and to gain a growth advantage. We first probed for a link between CRBP-I and differentiation by using a loss-of-function approach. Specifically, we compared the differentiation potential in three-dimensional reconstituted basement membrane (23) of MTSVWT and MTSVK° cells. Whereas MTSVWT underwent acinar differentiation in three-dimensional Matrigel, MTSVK° failed to differentiate and formed large, disorganized outgrowths (Fig. 3A, panels a and c). Transmission electron micrographs showed that MECSVWT formed typical epithelial cell–cell junctions, which were absent from MECSDK° colonies (Fig. 3B). Moreover, using Nile Red, a fluorescent lipid tracer, we showed that MECSVWT acini secreted lipids into the lumen and thus were functional (Fig. 3A, panels b and d). These results indicated that CRBP-I is involved in mammary epithelial cell differentiation in a genetic mouse model.

We next tested whether human SV40-immortalized MTSV1-7 cells could be induced to differentiate on ectopic CRBP-I expression. Cells transfected with empty vector (MTSVvector) did not express CRBP-I, and those transfected with CRBP-I (MTSVCRBP–I) expressed lower levels of CRBP-I protein than freshly isolated mammary epithelial cells, thus indicating that ectopic CRBP-I was not overexpressed (Fig. 3A, rightmost panel). Similar to MTSVK°, MTSVvector cells formed large, disorganized outgrowths in Matrigel, in contrast, and similar to MECSVWT cells, MTSVCRBP–I cells formed acinar structures that were competent in lipid secretion (Fig. 3A, panels e–h, cf. Fig. 3A, panels a–d). Hematoxylin and eosin staining confirmed that only MTSVCRBP–I had differentiated into hollow acini (Fig. 3C). We also observed that MTSVCRBP–I cells in monolayer culture underwent normal contact growth arrest in association with well-defined E-cadherin cell–cell junctions, whereas
MTSV\textsuperscript{vector} cells tended to pile up and had poorly defined E-cadherin complexes (Fig. 3D).

These results, based on reciprocal genetic approaches utilizing mouse and human mammary epithelial cells and multiple cell differentiation indices, strongly support the hypothesis that loss of CRBP-I function allows tumor cells to escape differentiation and progress toward increased autonomy. CRBP-I did not alter SV40 T/t antigen expression (data not shown), thus excluding this possible explanation for the above findings.

We anticipated that the prodifferentiation effect of CRBP-I was mediated by its ability to promote retinol storage and RAR activation. Indeed, treatment of MTSV\textsuperscript{CRBP-I} cells with 0.5 \textmu M Ro 41-5253, a selective RAR\(\alpha\) antagonist (24), blocked acinar differentiation, although some differentiated features (smooth colony borders) remained (Fig 4A). Conversely, long-term treatment of MTSV\textsuperscript{vector} cells with 0.5 \textmu M RA induced acinar differentiation (Fig. 4B). Conditions that precluded retinol storage and RAR activation (CRBP-I L29A and R58E mutants and overexpression of LRAT C161A) also precluded acinar differentiation (Supplementary Figure, which can be viewed online at http://jncicancerspectrum.oupjournals.org/jnci/content/vol97/issue1/), thus implicating increased retinol storage as an intermediate step in CRBP-I’s prodifferentiation role.

**CRBP-I and Tumorigenicity In Vivo**

The working hypothesis that emerged from the above findings was that somatic CRBP-I silencing might prevent tumor cells from taking up, storing, and using retinol, thus rendering tumors chronically deficient in RAR activity and prone to tumor progression. To test this concept in vivo, we inoculated athymic mice with \(1 \times 10^6\) MTSV\textsuperscript{vector} or MTSV\textsuperscript{CRBP-I} cells. Because these mice were WT for the CRBP-I locus and were fed a vitamin A–sufficient diet, any growth advantage of MTSV\textsuperscript{vector} cells would relate to a local deficiency in retinol bioactivity. Seven days after inoculation, tumor size (median 50; range 25–103) in the 12 mice that received MTSV\textsuperscript{vector} cells was greater than in the 12 mice that received MTSV\textsuperscript{CRBP-I} cells (median 18, range 0–28, Fig. 5A), and this difference was statistically significant. This growth differential increased over the next 3 weeks, with 10 of 12 MTSV\textsuperscript{vector} tumors continuing to grow or maintain their size and all 12 MTSV\textsuperscript{CRBP-I} tumors regressing to the point of being no longer palpable. Histologic sections stained with hematoxylin and eosin confirmed this large
Fig. 4. Cellular retinol-binding protein-I (CRBP-I) function in epithelial differentiation through retinoic acid receptor activation. A) Immortalized mammary epithelial cell lines transfected with CRBP-I (MTSV-CRBP-I) were treated for 48 hours with ethanol (EtOH) vehicle or 0.5 μM Ro 41-5253, plated in three-dimensional Matrigel, and treatment was continued for an additional 7 days. B) MTSV cells transfected with vector (MTSV-vector) were treated for 10 days with EtOH vehicle or 0.5 μM retinoic acid (RA), plated in three-dimensional Matrigel, and treatment was continued for 7 days. Panels show phase contrast micrographs of the outgrowths formed; scale bar, 100 μm (top) and 20 μm (bottom).

The difference in tumor growth and suggested that MTSV-vector cells actively invaded neighboring tissues (Fig. 5B).

DISCUSSION

Our results are of note on several grounds. First, we established that CRBP-I localizes primarily to lipid droplets, thus strengthening the notion that a major function of CRBP-I is the promotion of retinol storage in these organelles. Second, we established a link between a target cell’s ability to store retinol and its ability to use retinol to locally activate RARs under physiologic conditions; i.e., when cells are presented with retinol complexed to its plasma carrier protein as opposed to pharmacologic RA. Third, we established that CRBP-I, acting via its effect on retinol storage and through the downstream activation of RAR, promotes breast epithelial cell differentiation and growth inhibition, both in vitro and in vivo, which is consistent with earlier work implicating RARs in breast epithelial cell differentiation (25,26). Taken together, our data support the working hypothesis that the somatic loss of CRBP-I function in human (breast) cancer is an event that contributes to tumor progression by chronically depressing RAR activity and allowing tumor cells to escape differentiation and gain greater growth autonomy.

Our work should be viewed in the context of earlier work by several laboratories demonstrating that lecithin retinol acyltransferase, the chief enzyme catalyzing the esterification of retinol, is downregulated in cancers relative to corresponding normal tissue (27–29). Because retinol, when it is bound to CRBP-I, is the optimal substrate for lecithin retinol acyltransferase both in vitro (30,31) and in vivo (6), the lower activity of lecithin retinol acyltransferase likely would elicit the same phenotype as that of lowered CRBP-I function. Our finding that overexpression of the catalytically inactive C161A LRAT mutant blocked the effect of ectopic CRBP-I on RAR activity (Fig. 2E) and differentiation (Supplementary Figure, which can be viewed online at http://jncicancerspectrum.oupjournals.org/jnci/content/vol97/issue1/) is consistent with but does not confirm this expectation. Therefore, our work and that of previous investigators together suggest that the decreased expression of either lecithin retinol acyltransferase or CRBP-I in human cancers may compromise retinol storage and, as demonstrated here, RAR activation. We have not addressed the important question of whether the co-functional role of lecithin retinol acyltransferase and CRBP-I is associated with the localization of lecithin retinol acyltransferase to lipid droplets, as demonstrated here for CRBP-I. Other related topics that remain to be explored are the effect, if any, of CRBP-I or lecithin retinol acyltransferase on the activity of the retinyl ester hydrolases that catalyze the deesterification of retinol (32–34), or the effect of CRBP-I or lecithin retinol acyltransferase, if any, on the expression of the cellular RA-binding proteins I and II, which regulate RA transport and metabolism (35–37).

The loss of CRBP-I function is a frequent but not universal event in human (breast) cancer (8–11), implying that a cohort of cancers progress regardless of CRBP-I expression status. This independence may be because in tumors that express CRBP-I the oncogenic process is driven by a pathway that is not antagonized by or overwheels CRBP-I action. For instance, epigenetic CRBP-I silencing has been associated with myc—not erbB2-driven mouse mammary tumorigenesis (38). Alternatively, retinol metabolism/RAR activation may be disabled in CRBP-I-expressing tumors through the (epi)genetic inactivation of other genes whose products are required for retinol metabolism and RAR activation. Reports of cancer-specific reduced expression of genes encoding lecithin retinol acyltransferase (see above), RA-synthesizing enzymes, and RAR species lend support to this notion (2,27–29,39).

Two independent approaches used in this study (CRBP-I and lecithin retinol acyltransferase point mutants) implicate retinol storage as an integral link between CRBP-I and RAR activation. Further work is required, however, to better define the mechanism of this link. We speculate that in our model system, the physiologic uptake of retinol may be obligatorily tied to its esterification (40), which is dependent on CRBP-I and lecithin retinol acyltransferase; oxidation to RA would then proceed following deesterification. A recent report demonstrated that retinol storage cells in the lung function in RA synthesis, but the study did not specifically address the role of CRBP-I (41). The ability of an RARα-selective antagonist to partially block the effect of CRBP-I (Fig. 4A) suggests that RARα, which is expressed in MTSV1-7 cells (42), partially mediated the CRBP-I effect in our model. RARα2 and RARβ2 are induced by RA in MTSV1-7 cells (42), and this activity would be expected to be indirectly increased by CRBP-I. Despite an earlier attempt (7), this expectation remains to be conclusively tested.
CRBP-I has been genetically implicated in the maintenance of normal circulating levels of vitamin A when its dietary intake is limited but otherwise has been found to be dispensable for health (6). Our in vivo data suggest that somatic CRBP-I loss of function results in a local deficit in vitamin A storage and metabolism that has profound consequences for the affected tissue, despite presumably normal circulating levels of vitamin A. The consequence of deficient vitamin A storage is a fundamental point that needs to be studied further, but if CRBP-I is indeed essential both centrally (to maintain liver retinol stores and thus maintain circulating retinol levels) and at the target tissue level (to promote RAR activity and epithelial differentiation), then epidemiologic studies attempting to correlate human vitamin A status with cancer incidence may have been partly misguided. Undoubtedly, vitamin A hypovitaminosis may compromise all biologic effects of the vitamin, but normal or even excess vitamin A levels may still be associated with premalignant or malignant disease if the cells that require retinol storage to function have disabled genes such as CRBP-I or LRAT and are thus unable to store and metabolize retinol appropriately. It remains possible, however, that vitamin A hypovitaminosis will enhance the severity of somatic CRBP-I silencing (9). These hypotheses are amenable to direct in vivo testing using genetically engineered mice. Because CRBP-I and LRAT positively regulate RAR activity by regulating metabolism of the proligand retinol, tumor progression resulting from CRBP-I or LRAT inactivation could, in principle, be antagonized by pharmacologic treatment with RAR ligands that do not require prior metabolic activation.

REFERENCES

(15) Wu CC, Howell KE, Neville MC, Yates JR, 3rd, McManaman JL. Proteomics reveal a link between the endoplasmic reticulum and lipid secre-

Fig. 5. Effect of cellular retinol-binding protein-I (CRBP-I) on tumor growth in vivo. A) Immortalized mammary epithelial cell lines transfected with vector (MTSVvector) or WT CRBP-I (MTSVCRBP-I) were inoculated in vivo, and tumor growth was followed as described in Methods. Median tumor volume and individual data points (n = 12) are plotted over time (12 of 12 MTSVCRBP-I tumors regressed completely or nearly so by day 30; 2 of 12 MTSVvector tumors regressed by day 30). Data are for one representative experiment of two performed. * = P < .001, two-sided Mann–Whitney U test, n = 12 mice per group. B) Hematoxylin and eosin–stained paraffin thin sections of tumors formed by MTSVvector (panels a, c) and MTSVCRBP-I cells (panels b, d). Letter T in panel (a) designates a relatively large tumor mass that is compressing the skin; arrows in panels (b) and (d) point to small residual tumor cell clusters; panel (c) illustrates tumor cell interdigitation into adjacent muscle tissue, with the arrow pointing to tumor cells, which are more darkly stained. Scale bars, 200 μm (panels a, b), 100 μm (c, d).


NOTES

Present address: Yuvarani S. Kuppumbatti, Allivio, Inc., Lake Forest, California.

We thank P. Chambon, J. Taylor-Papadimitriou, D. Teng, R. Lotan, J. Reddy, M. Klaus, R. Krauss, and D. Bok for reagents and L. Ossowski and J. Aguirre-Ghiso for much helpful advice. This work was supported by National Cancer Institute grant R01 CA54273 to RML, the Samuel Waxman Cancer Research Foundation (SWCRF), The Norman and Rosita Winston Foundation, NCI shared resources grant R24 CA95823 to Mount Sinai School of Medicine’s Microscopy Shared Resource Facility, and National Cancer Institute shared resources grant R24 CA88302 to Mount Sinai School of Medicine’s Mouse Genetics Shared Research Facility. RML is a SWCRF investigator.

Manuscript received May 28, 2004; revised October 12, 2004; accepted November 1, 2004.