

Mammary Carcinoma Suppression by Cellular Retinoic Acid Binding Protein-II¹

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ABSTRACT

Retinoic acid (RA) modulates cell proliferation, differentiation, and apoptosis, and is used in chemotherapy and chemoprevention in several human cancers. RA exerts its pleiotropic activities by activating the nuclear receptors, retinoic acid receptor (RAR), which, in turn, regulate transcription of multiple target genes. In cells, RA also associates with cellular RA-binding proteins [cellular RA binding proteins (CRABPs)-I and -II]. Recent studies revealed that CRABP-II functions by “channeling” RA to RAR, thereby enhancing the transcriptional activity of the receptor. In search for a biologically meaningful role for CRABP-II, we examined its effect on RA-induced growth inhibition in RA-resistant tumors. Stable expression of CRABP-II in mammary carcinoma SC115 cells enabled activation of RAR, considerably sensitized the cells to RA-induced growth inhibition, and dramatically suppressed their tumorigenicity in immunodeficient mice. Similarly, injection of an adenovirus expressing CRABP-II into mammary carcinomas that spontaneously develop in TgN(*MMTVneu*)202Mul mice resulted in a significant delay in tumor growth and in prolonged survival rates. Remarkably, in both mouse models, administration of exogenous RA had no additional beneficial effect, indicating that endogenous levels of RA are sufficient for maximal tumor suppression on CRABP-II overexpression. The observations reveal that CRABP-II plays a critical role in sensitizing tumors to the growth-suppressive activities of RA *in vivo*.

INTRODUCTION

The vitamin A metabolite all-*trans*-RA³ is a potent modulator of cell growth, differentiation, and apoptosis in a variety of tissues both in the embryo and in the adult. RA and related molecules also display growth-inhibitory activities in some carcinomas, and are used as chemotherapeutic and chemopreventive agents in a variety of malignancies such as head and neck cancers, promyelocytic leukemias, and cancers of the respiratory and digestive tracts (1–6). RA was also suggested to be efficacious in the treatment of breast cancer. Retinoids were shown to inhibit growth of mammary carcinomas in cultured cells (7–12), in mammary gland organ cultures (13), in mammary carcinoma xenografts in immunocompromised mice (7), and in transgenic mammary cancer mice models (1, 3). However, retinoid therapy is confounded by the toxicity of these agents at pharmacological doses (1, 14) and by the development of RA-resistance (15–17). In attempts to overcome these problems, numerous retinoids have been synthesized and studied over the past 20 years in search for compounds with higher efficacy and lower toxicity. However, these efforts have been

only partially successful, and the search for “better retinoids” and for novel approaches to increase the therapeutic index of RA is ongoing.

Whereas the precise molecular mechanisms underlying RA-induced growth inhibition remain by and large unknown, it is well established that, in many cells, these activities involve modulation of transcriptional rates and are mediated by RARs, ligand-inducible transcription factors that are members of the superfamily of nuclear hormone receptors (18, 19). In addition to nuclear receptors, RA binds in cells to two intracellular lipid-binding proteins, termed CRABPs (CRABP-I and CRABP-II). CRABPs are 15 kDa proteins that bind RA with a high affinity and selectivity (20, 21). The two CRABPs display markedly different expression profiles across tissues and developmental stages, and a high level of evolutionary conservation, suggesting that they have not only important, but distinct, functions in mediating the biological effects of retinoids. Indeed, recent studies revealed that expression of CRABP-II, but not of CRABP-I, enhances the transcriptional activity of RAR α (22–25). Regarding the mechanism through which CRABP-II modulates the activity of RAR, we showed that whereas CRABP-II is predominantly cytosolic in the absence of ligand, it responds to RA by translocating to the nucleus, where it can associate directly with RAR. We demonstrated additionally that the CRABP-II·RAR complex mediates ligand “channeling” from the binding protein to the receptor, thereby facilitating the ligation of RAR and potentiating its transcriptional activity (22–24). Despite these mechanistic insights into the cooperation between CRABP-II and RAR, the biological significance of this interaction remained uncertain in light of reports that CRABP-II^{-/-} mice are viable, fertile, and display only minor aberrations (26).

The present study was undertaken in search for a clear indication for a biologically meaningful role for CRABP-II. Thus, we examined the effect of expression of this protein on RA-induced growth inhibition in tumors. We hypothesized that if CRABP-II enhances the activity of RAR, then overexpression of the binding protein will sensitize cells to the growth-inhibitory activity of RA and may reverse the RA resistance of some tumors. Two RA-resistant breast cancer models were used in these studies. In one, the tumorigenicity of mammary carcinoma SC115 cell lines that express different levels of CRABP-II was investigated in immunodeficient mice. In a second set of experiments, an adenovirus construct expressing CRABP-II was injected into mammary carcinomas that develop spontaneously in the transgenic mice TgN(*MMTVneu*)202Mul, engineered for mammary-specific overexpression of the *neu* gene. The data demonstrate that expression of CRABP-II dramatically suppresses tumor development in both models. Notably, expression of CRABP-II was sufficient to inhibit tumor growth even in the absence of exogenous RA administration.

MATERIALS AND METHODS

Ligands. RA was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Stock solutions were made in DMSO and stored in amber vials at –80°C.

Cell Lines. SC115 cells and their clones were generously provided by Rafael Mira-y-Lopez (Mount Sinai School of Medicine, New York, NY). Cells were cultured in DMEM supplemented with 10% fetal bovine serum. Stably

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³ The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; CRABP, cellular retinoic acid-binding protein; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdUrd, bromodeoxyuridine; RT-PCR, reverse transcription-PCR; Ad, adenovirus.

transfected cell lines were also supplemented with 500 $\mu\text{g/ml}$ G418 (neomycin; Clontech, Palo Alto, CA).

Antibodies. Antibodies against CRABP-II (5CRA3B3) were a generous gift from Pierre Chambon (IGMCB, Strasbourg, France). Antibodies against actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse and antigoat immunoglobulin-horse radish peroxidase antibodies were purchased from Amersham (Arlington Heights, IL). Primary and secondary antibodies for Western blots were diluted 1:1000 and 1:3000, respectively.

MTT Assays. Cells were seeded in 96-well plates (500 cells/well) and allowed to grow for 24 h. RA dissolved in DMSO or DMSO alone was added at the indicated concentrations (solvent concentration $<0.1\%$). Cells were refed every 48 h. On the day of the assay, cells were treated with 10 μl MTT reagent (Roche Diagnostics) and incubated for 4 h. After the addition of 100 μl of solubilization buffer (Roche Diagnostics), cells were incubated overnight, and absorbance at 570 nm was measured.

Transactivation Assays. SC115 cells or their derivatives were cotransfected with a DR5-tk-luciferase reporter plasmid and pCHI110 vector harboring the β -galactosidase cDNA (internal standard) using the Fugene6 reagent according to manufacturer protocol (Roche Diagnostics, Indianapolis, IN). Twenty-four h after transfection, cells were treated with RA for 24 h and lysed. Luciferase expression was measured using the luciferase assay system (Promega) according to the manufacturer's protocol. Reporter activity was normalized for transfection efficiency using β -galactosidase.

Adenoviruses. Human CRABP-II cDNA was amplified and two restriction sites added (5' *EcoRI* and 3' *BamHI*) by PCR. The product was ligated into the shuttle vector pAD5 CMV k-Npa, and recombinant adenovirus was prepared by the Gene Transfer Vector Core, University of Iowa (Iowa City, IA), as described (27). Viruses (2.5×10^6 infectious particles per ml in PBS) were stored at -80°C until use.

Experimental Animals. Mice were housed at the Cornell Veterinary College barrier facility. Animal care and use was approved by the Institutional Animal Care and Use Committee.

Xenograft Experiments. Four to 5-week-old CD-1 nude athymic female mice (Charles River Laboratories) were injected with 2×10^6 SC115 cells or clones that stably overexpress CRABP-II. Parental and CRABP-II-overexpressing cells in PBS were injected into the right and the left mammary fat pad of the same animal, respectively. RA was dissolved in refined sesame oil (Sigma Chemical Co.) and administered to the mice by direct pipetting into the mouth (50 $\mu\text{l}/\text{dose}$). Mice were treated p.o. with 2 or 8 mg RA/kg/day (or sesame oil alone) 5 days a week. Tumor growth was monitored for 6 weeks, at which point the experiment was terminated and tissues collected for pathology evaluation. Resected tumors were measured in three dimensions with a caliper, and their volume calculated using the formula: $V = \pi/6(L \times W \times H)$.

Adenovirus Administration. Adenoviral delivery was performed by direct intratumoral injection with a Hamilton syringe and a 30-gauge beveled needle after deep anesthesia with i.p. Avertin (2.5% v/v in 0.9% NaCl; 0.015–0.020 ml/g body weight). Tumors with volume 0.260 cm^3 were injected with 3×10^7 pfu/ $0.5 \mu\text{l}/\text{mm}^3$ tumor volume adenovirus harboring CRABP-II cDNA or with "empty" recombinant adenovirus (Ad0), serving as control. According to our earlier experiments with adenovirus harboring LacZ, this volume is sufficient for diffusion through the entire volume of a tumor of this size (28).

Carcinogenesis Studies in the MMTV-neu Mice. A colony of the homozygous strain TgN(MMTVneu)202Mul mice (29) was maintained on FVB/N background. All of the experiments were performed on virgin females. Tumor development was monitored by palpation twice a week, and tumor sizes were recorded without knowledge of the applied treatment. RA was administered by 21-day release 5-mg pellets (Innovative Research of America, Sarasota, Florida) implanted s.c.

Pathological Analyses. Mice were anesthetized with avertin, and, after cardiac perfusion at 90 mm Hg with PBS followed by phosphate-buffered 4% paraformaldehyde, mammary glands with tumors were removed and characterized by microscopic evaluation of paraffin sections stained with H&E. Lesions were identified according to the Classification of Neoplasia of Genetically Engineered Mice endorsed by the Mouse Models of Human Cancer Consortium (30). Rates of cell proliferation and apoptosis were estimated according to earlier established protocols (31). Briefly, representative sections from tumors were scored for BrdUrd uptake and apoptosis. At least 150 cells per field in 10 fields were scored for each sample.

PCR and RT-PCR Assays. Tumor cells were isolated from frozen sections using a Laser Microdissection System (Leica). DNA was isolated as described (32). mRNA was prepared by purification with Absolutely RNA Nanoprep kit (Stratagene). Reverse transcription with 13 μl of eluate and subsequent PCR were performed essentially as described (33, 34). The primers for CRABP-II cDNA were 5'-AGC AGT GGA GAT CAA ACA GGA GGG A-3' (CRABP-II hex2-5', sense), 5'-CAG CAG TCG AGA TCA AAC AGG AGA A-3' (CRABP-II mex2-5', sense), and 5'-AAG TTC CCA CTC TCC CAT TTC ACC A-3' (CRABP-II uex3-3', antisense). Primers CRABP-II hex2-5' and CRABP-II mex2-5' are specific for human and mouse exon 2, respectively. The primer CRABP-II uex3-3' recognizes both human and mouse sequence in exon 3. Amplification of genomic and cDNA results in 345-bp and 159-bp PCR fragment, respectively. To minimize sample-to-sample variations resulting from RNA degradation and pipetting error, β -actin was used as an internal standard. β -Actin transcripts were detected with primers AC6 (5'-TGG GTA TGG AAT CCT GTG GCA TCC A3', sense) and AC5 (5'-GAG GGG CCG GAC TCA TCG TAC TC3', antisense) as described (35).

Statistical analyses were carried out using InStat 3.03 and Prism 3.02 (GraphPad, Inc., San Diego, CA) software. Survival fractions were calculated using the Kaplan-Meier method. Survival curves were compared by log rank Mantel-Haenszel tests. Means were compared by estimation of the two-tailed P with unpaired t tests.

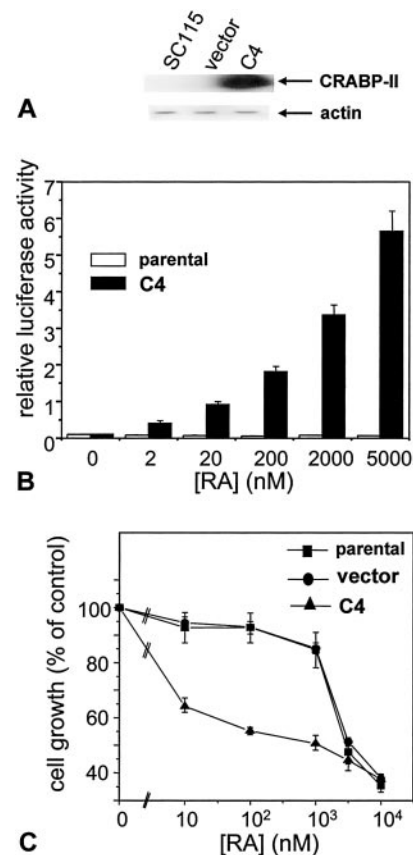


Fig. 1. CRABP-II enhances the transcriptional activity of RAR and sensitizes SC115 cells to RA-induced growth inhibition. **A**, Western blot analysis of CRABP-II expression in parental SC115 cells, and in clones stably expressing either an empty vector (*vector*) or a CRABP-II construct (*C4*). Fifteen μg of total cell lysate protein was loaded in each lane, and blots were probed for actin to ensure equivalent loading. **B**, cells were cotransfected with a luciferase reporter construct driven by a RAR element along with a pCHI110 vector (internal standard). Cells were treated with RA for 24 h, lysed, and luciferase activity was measured and normalized for β -galactosidase activity. Data are presented as fold induction relative to cells treated with vehicle alone. Bars represent means \pm SE ($n = 3$). **C**, MTT growth assays. SC115 parental and their derivatives were treated with RA for 7 days, and viable cell number was scored with the MTT assay. Data represent cell growth as a percentage of vehicle-treated cell growth and are mean ($n = 5$); bars, \pm SE.

Table 1 Number and volume of tumors arising from SC115 cells injected into mammary fat pads of CD-1 mice

Four week-old CD-1 nude female mice were used. Parental SC115 cells or their derivative overexpressing CRABP-II (clone 4) were injected into the right and the left mammary fat pad of the same animal, respectively. RA (0, 2, or 8 mg/kg body weight/day) was administered orally 5 days a week, as described in "Materials and Methods." Tumor volumes are given as mean \pm SE. Sites injected with CRABP-II-overexpressing cells gave rise to fewer tumors (57%; $P = 0.0013$), and these tumors were markedly smaller (12%; $P < 0.0001$).

RA (mg/kg/day)	SC115 cells		SC115 cells overexpressing CRABP-II	
	No. of tumors/ no. of injection sites	Tumor volume (mm ³ , mean \pm SE)	No. of tumors/ no. of injection sites	Tumor volume (mm ³ , mean \pm SE)
0	7/7	99 \pm 14	3/7	13 \pm 1
2	6/6	189 \pm 6	4/6	65 \pm 9
8	8/8	158 \pm 11	5/8	26 \pm 4
Combined	21/21	147 \pm 19	12/21	19 \pm 6

RESULTS

Expression of CRABP-II Is Essential for the Transcriptional Activity of RAR in SC115 Mammary Carcinoma Cells. The involvement of CRABP-II in cellular responses to RA was investigated using the androgen-sensitive mammary carcinoma cell line SC115, which was reported to be RA-resistant (36). To investigate the effect of CRABP-II expression on RA activity, several derivatives of these cells were used: parental SC115 cells, in which expression of CRABP-II is below the level of detection by Western blots, SC115 derivatives that stably overexpress CRABP-II, and a line stably transfected with an empty vector, which was used as a control (Fig. 1A). These cell lines were transiently transfected with a luciferase reporter construct driven by the response element

DR-5, which specifically binds RAR-retinoid X receptor heterodimers (18), and the ability of RA to induce expression of the reporter was studied (Fig. 1B). Strikingly, RA did not elicit an RAR-mediated transcriptional activity in the parental SC115 cells even at high ($>5 \mu\text{M}$) RA concentrations. Stable overexpression of CRABP-II dramatically enhanced the transcriptional activity of RAR at all of the ligand concentrations used, and enabled transcriptional activation to ensue at nM concentrations. These observations demonstrate that SC115 cells express the necessary components for proper RA-induced, RAR-mediated, transcriptional responses, *i.e.*, RAR, retinoid X receptor, and their associated coactivators. Nevertheless, no RA-induced transcriptional activity can be observed in these cells unless CRABP-II is expressed.

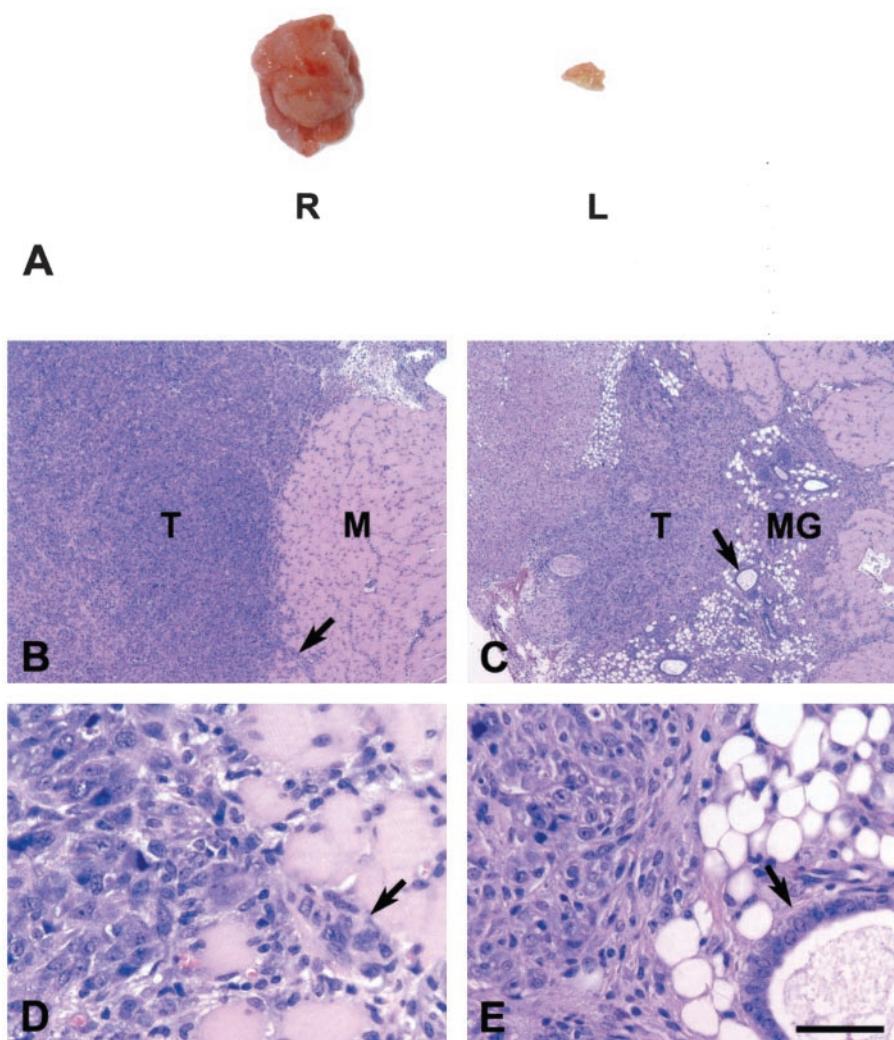


Fig. 2. CRABP-II inhibits tumor development in a xenograft mouse model. Four-week-old CD-1 nude female mice were injected with parental SC115 cells or SC115 cells that stably overexpress CRABP-II (clone 4) into the right and the left mammary fat pad, respectively. Tumor growth was monitored for 6 weeks, at which point the experiment was terminated. A, gross pathology of tumor nodes dissected from the right (R) and left (L) mammary fat pad. Note the difference in size between tumors formed by parental SC115 cells (R) and stably overexpressing CRABP-II clone (L). B and D, large tumor formed by parental SC115 cells. The tumor (T) consists of highly mitotic epithelioid cells invading (arrows) surrounding muscles (M). C and E, small tumor mass (T) formed after injection of cells stably overexpressing CRABP-II into the mammary fat pad (MG). Note residual mammary gland structures in the tumor vicinity (arrows; B-E). Staining with H&E. Bar: A, 6 mm; B and C, 400 μm ; D and E, 40 μm .

CRABP-II Sensitizes SC115 Cells to RA-induced Growth Inhibition. The critical need for CRABP-II for the transcriptional activity of RAR in SC115 cells raises the possibility that the lack of this protein may underlie their RA-resistance, and thus, that ectopic expression of CRABP-II may sensitize them to RA-induced growth inhibition. The ability of RA to inhibit growth of the SC115 cell lines that express different levels of CRABP-II was assessed by MTT cell growth assays. RA induced growth inhibition in all of the cell lines, but displayed markedly different dose-response characteristics (Fig. 1C). Parental SC115 cells as well as the vector-transfected controls were refractive to RA up to μM concentrations, demonstrating their RA resistance. On the other hand, SC115 cells that overexpress CRABP-II were markedly more sensitive to RA-induced growth inhibition, displaying an EC_{50} of ~ 10 nM. Hence, overexpression of CRABP-II was accompanied by a 2 orders of magnitude increase in the RA sensitivity of the cells. To verify that these observations did not stem from clonal variations, we tested additional CRABP-II overexpressing clones, and obtained similar results (data not shown).

CRABP-II Inhibits the Tumorigenicity of SC115 Cells in a Xenograft Mouse Model. The ability of CRABP-II to sensitize carcinoma cells to the tumor-suppressive activities of RA was additionally investigated *in vivo* using an immunodeficient mouse model. The different SC115 clones were injected into the mammary fat pads of CD1 “nude” mice. To minimize variability between animals, one mammary fat pad (right side) in each animal was injected with the parental SC115 cells and the other pad (left side) injected with SC115 cells that stably overexpress CRABP-II. All of the sites injected with the parental SC115 cells developed tumors during the experiment (Table 1). Sites injected with cells that express CRABP-II showed a marked decrease in tumor occurrence (57%; Fisher’s exact test; $P = 0.0013$). Importantly, tumors that developed from CRABP-II-overexpressing cells were significantly smaller (7-fold; $P < 0.0001$), as evidenced by visual inspection (Fig. 2A) and by volume measurements (Table 1) of the resected tumors. Histological evaluation of SC115 tumor specimens revealed that they consist of epithelioid cells with numerous mitoses and infiltrate adjacent muscle tissue (Fig. 2, B and D). SC115-CRABP-II xenografts formed smaller, if any, tumors, which localized in the mammary fat pad (Fig. 2, C and E). These data conclusively demonstrate that expression of CRABP-II, in addition to inhibiting the growth of cultured cells, leads to pronounced inhibition of SC115 tumor growth *in vivo*. Strikingly, CRABP-II exerted its antitumorigenic activity even in the absence of RA treatment. Daily treatment of the animals with RA had no beneficial effects on the tumorigenicity (reflected by either tumor incidence or tumor volume) of either cell-line (Table 1). We note that RA treatment appeared to somewhat facilitate tumor growth but that the effect was not statistically significant. These data suggest that the CRABP-II effect is saturated at endogenous RA concentrations.

CRABP-II Inhibits Mammary Carcinoma Growth in the MMTV-neu Transgenic Model. To explore the possibility that CRABP-II may be effective in inhibiting tumor progression in an animal model that better mimics human cancer, we used the well-characterized TgN(MMTV-neu)202Mul transgenic mouse (29, 37). The oncogenic hallmark of this model is a mammary-specific overexpression of the receptor protein tyrosine kinase Erb-B2/neu/HER-2. Constitutive expression of Erb-B2 occurs, as a result of gene amplification and/or overexpression, in a large percentage of primary human breast cancers (38), and its presence is inversely correlated with long-term survival in human patients (39–41). Interestingly, overexpression of *neu* in mammary carcinoma cells was shown to lead to RA resistance (42). In the MMTV-neu mouse model, 100% of female mice develop mammary adenocarcinomas with a median time for tumor

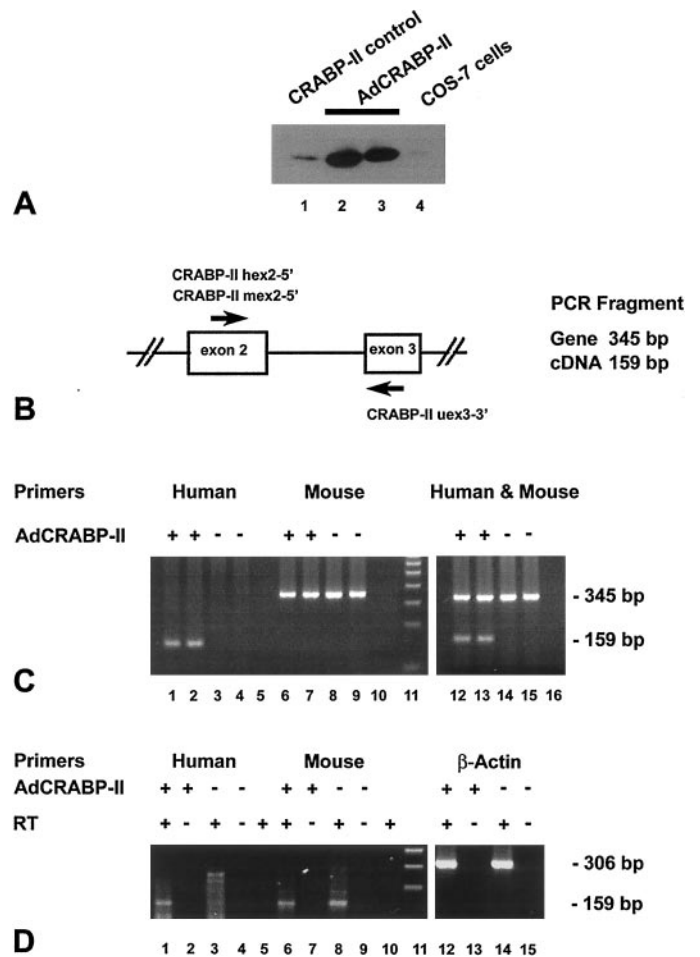


Fig. 3. Adenovirus-mediated expression of CRABP-II. A, COS-7 cells were cultured in 100-mm plates, transfected with $50 \mu\text{l}$ (3×10^9 pfu) of adenovirus harboring human CRABP-II cDNA, incubated for 24 h, and lysed (Lanes 2 and 3). Bacterially expressed CRABP-II was used as a control (Lane 1). Lysates ($8 \mu\text{g}$ protein/lane) were resolved by SDS-PAGE, and CRABP-II visualized by a Western blot. B, design of primers for discriminative identification of human and mouse CRABP-II sequences. Oligonucleotides (5') are specific either for the human (CRABP-II hex2-5') or the mouse (CRABP-II mex2-5') CRABP-II sequences in the second exon. The 3' oligonucleotide recognizes exon 3 of both the human and the mouse sequence, and its use results in equal priming efficiency of both human and mouse mRNA during reverse transcription. According to the CRABP-II gene structure (57), amplification of the gene and the cDNA will result in 354-bp and 159-bp fragments, respectively. C, primer-specific detection by microdissection and PCR analysis of the human CRABP-II transgene in mammary tumor cells treated with AdCRABP-II (Lanes 1, 2, 6, 7, 12, and 13), but not in those from tumors exposed to blank adenovirus (Ad0, Lanes 3, 4, 8, 9, 14, and 15). Lanes 5, 10, 16, PCR without DNA input. Lane 11, DNA size markers (100-bp ladder). D, detection of human and mouse CRABP-II mRNAs (Lanes 1, 3 and 6, 8, respectively; 159 bp) and β -actin (Lanes 12 and 14, 306 bp) in the mammary tumor cells treated either with AdCRABP-II (Lanes 1, 2, 6, 7, 12, and 13) or Ad0 (Lanes 3, 4, 8, 9, 14, and 15). Lanes 2, 4, 7, 9, 13, and 15, RT-PCR reactions without reverse transcriptase (RT-). Lanes 5 and 10, RT-PCR without RNA input Lane 11, DNA size markers (100-bp ladder). C and D, 3% NuSieve agarose gels stained with ethidium bromide.

progression at ~ 205 and 237 days for multiparous and virgin females, respectively (29).⁴

For localized delivery of the gene, hCRABP-II cDNA was placed into a recombinant replication-deficient adenoviral vector as described in “Materials and Methods.” Infection of cultured COS-7 cells with this virus resulted in high level of expression of the protein (Fig. 3A). The recombinant adenovirus (AdCRABP-II) or a control, empty, virus (Ad0) were used to directly infect mammary tumors that arise in virgin female MMTV-neu mice. Successful adenovirus-mediated expression of CRABP-II in the tumors was verified by PCR-

⁴ A. Y. Nikitin, unpublished observations.

mediated detection of adenoviral DNA (Fig. 3, B and C) and mRNA (Fig. 3, B and D) in microdissected tumor specimens collected 3 days after infection. The amount of transgene mRNA was found to approach that of endogenous CRABP-II mRNA.

To monitor the physiological consequences of adenovirus-mediated expression of CRABP-II, tumor sizes were measured in mice treated with either AdCRABP-II or Ad0 in the presence or absence of RA administration (Fig. 4). Tumors treated with AdCRABP-II exhibited a dramatic difference in the kinetics of tumor formation. Specifically, there was a >2-week delay in the initial increase of tumor volume, as

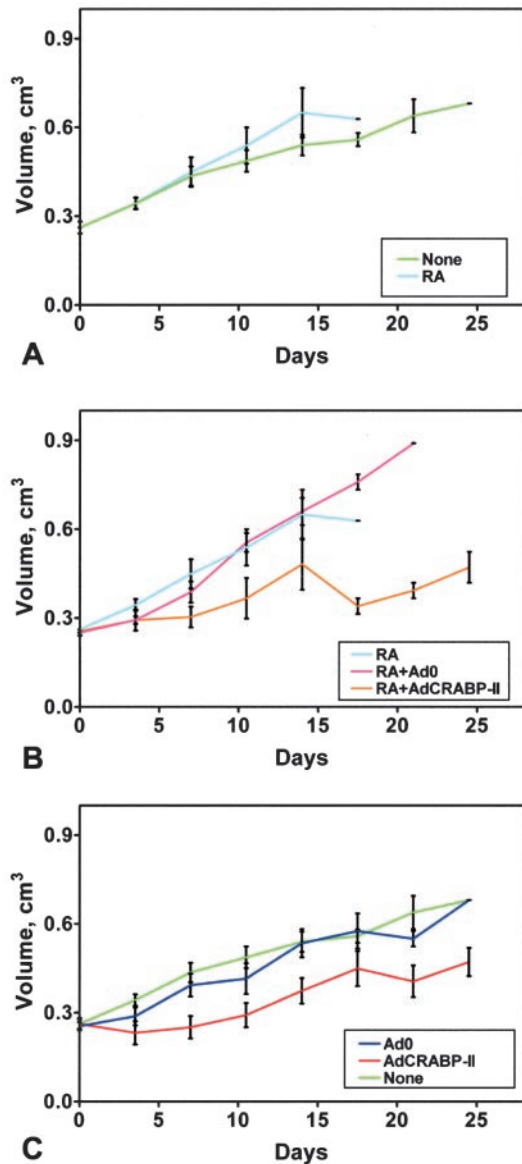


Fig. 4. Effect of CRABP-II and RA on tumor growth in *MMTV-neu* mice. Virgin female *MMTV-neu* mice were allowed to develop mammary tumors with volume 0.262 cm^3 (0.5 cm in diameter). At this point, mice were subjected to systemic RA administration (A and B) and/or intratumoral adenovirus injection (B and C) containing either human CRABP-II cDNA (*AdCRABP-II*) or blank adenovirus (*Ad0*) as described in "Materials and Methods." Sizes of tumors were measured with calipers twice a week. In mice treated with RA (B), on day 10 after injection, the volumes of tumors treated with *AdCRABP-II* were significantly smaller than tumors treated with an empty virus (mean: $0.3665 \pm 0.1527 \text{ cm}^3$; $n = 5$ versus $0.5550 \pm 0.07025 \text{ cm}^3$; $n = 5$; $P = 0.0365$). In mice without RA treatment (C) the volumes of tumors treated with *AdCRABP-II* were significantly smaller on day 7 (*AdCRABP-II* versus *Ad0*, mean \pm SD; $0.2506 \pm 0.1001 \text{ cm}^3$, $n = 7$ versus $0.3927 \pm 0.1014 \text{ cm}^3$, $n = 7$, $P = 0.0216$), and on day 14 (*AdCRABP-II* versus *Ad0*, mean \pm SD; $0.3740 \pm 0.1148 \text{ cm}^3$, $n = 7$ versus $0.5348 \pm 0.1263 \text{ cm}^3$, $n = 7$, $P = 0.0283$) after injection; bars, \pm SD.

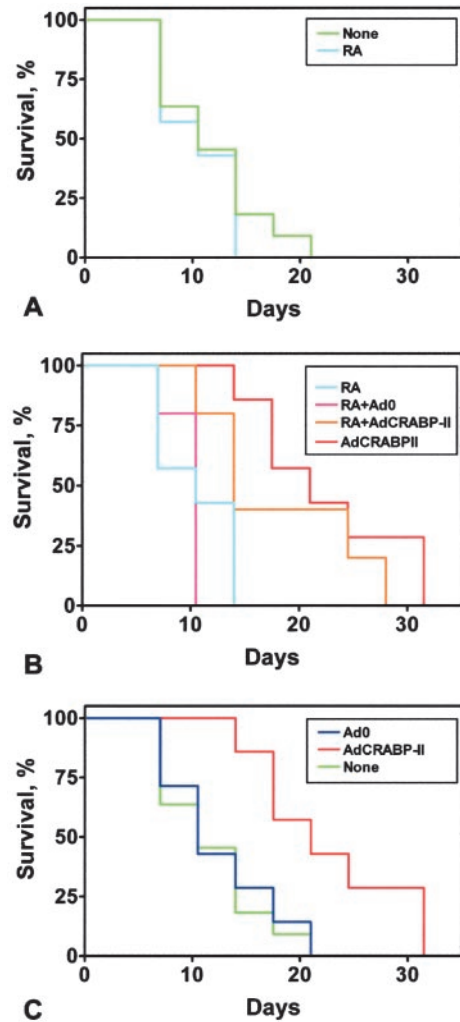


Fig. 5. Effects of CRABP-II expression and of RA treatment on survival of *MMTV-neu* mice. Mice were treated as described in the legend to Fig. 4, and the experiment was completed when the volume of mammary tumors reached 0.524 cm^3 . Median survivals were 10, 10, 10, 14, 10, and 21 days for mice with no treatment (none, $n = 11$), RA ($n = 7$), RA+*Ad0* ($n = 5$), RA+*AdCRABP-II* ($n = 5$), *Ad0* ($n = 7$), and *AdCRABP-II* ($n = 7$). $P = 0.0145$ and 0.0136 for log-rank comparisons of survival curves of mice treated with RA+*Ad0* versus RA+*AdCRABP-II* (B) and *Ad0* versus *AdCRABP-II* (C).

compared with control-adenovirus treatments (Fig. 4B). The transient nature of this delay is consistent with temporary expression of transgenes delivered by adenovirus. Interestingly, simultaneous administration of RA and *AdCRABP-II* had only marginal, if any, additional effect over that observed with *AdCRABP-II* alone.

To gauge the long-term effect of *AdCRABP-II* administration, mouse survival, defined as the time when tumor volume reached 0.524 cm^3 , was assessed (Fig. 5). Although all of the mice treated with *AdCRABP-II* eventually developed tumors of that size, their median survival was twice longer than that of mice treated with *Ad0* (21 versus 10 days; Fig. 5C). Additional RA administration was less beneficial, and median mouse survival for the RA+*AdCRABP-II* treatment regime was 14 days, as compared with 10 days for mice with RA+*Ad0* treatment (Fig. 5B). Taken together, these observations indicate that elevated levels of CRABP-II inhibit tumor growth *in vivo* and that the inhibitory effect is not improved on administration of exogenous RA.

CRABP-II Expression Results in Induction of Apoptosis in Tumors That Develop in *MMTV-neu* Mice. To better understand the mechanism through which CRABP-II exerts its antitumorigenic

activity, the effects of protein expression on several growth characteristics of the tumors were evaluated (Fig. 6). The fraction of apoptotic cells was significantly higher (~4-fold) in mammary tumors treated with AdCRABP-II (Fig. 6, A–C) both with and without RA treatment (Fig. 6C). Using BrdUrd incorporation to assess cell-cycle distribution showed that the fraction of mammary tumor cells in S phase of the cell cycle was not significantly affected by AdCRABP-II or RA administration (Fig. 6D). We note that, in the absence of RA treatment, the BrdUrd index was lower in animals treated with either AdCRABP-II or Ad0 (Fig. 6D). This decrease may be because of nonspecific effects of adenoviral infections on tumor cell proliferation. A modest effect of RA on proliferation of cells could provide an alternative explanation but, in view of the observations that RA does not affect tumor growth (Fig. 4A), this explanation is unlikely. Because tumor growth is governed mainly by the balance between cell proliferation and apoptosis, the data strongly suggest that the growth inhibition of mammary carcinomas in *MMTV-neu* mice treated with AdCRABP-II stems mainly from increased apoptotic rates.

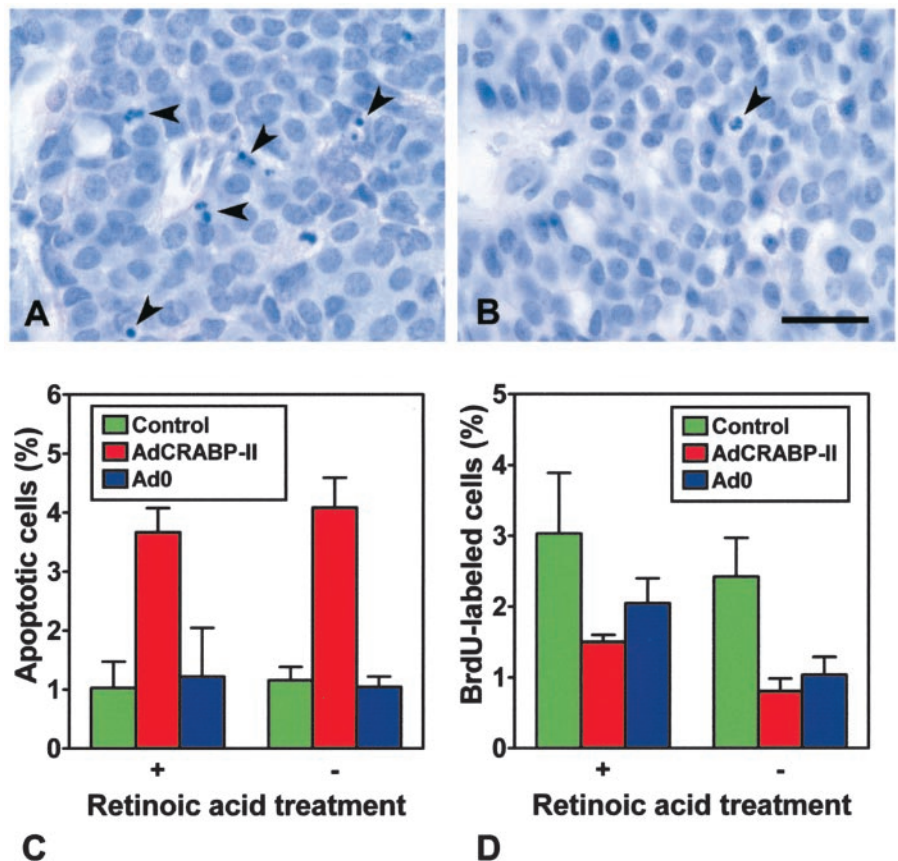
DISCUSSION

It was shown previously that CRABP-II functions by channeling RA to RAR, and that expression of this protein in cultured cells markedly enhances RA-induced, RAR-mediated, transcriptional activities (22–24). The goal of the present work was to explore whether expression of CRABP-II potentiates the ability of RA to suppress growth of mammary carcinomas. Hence, the effect of CRABP-II on RA-induced growth inhibition in the androgen-dependent mammary carcinoma SC115 cells was examined. These studies demonstrated that RA was unable to activate RAR in cultured SC115 cells and that this was accompanied by a marked RA-resistance. Expression of

CRABP-II in these cells considerably enhanced the transcriptional activity of RAR and dramatically sensitized the cells to RA-induced growth inhibition (Fig. 1). Correspondingly, expression of CRABP-II in SC115 cells dramatically reduced their tumorigenic potential in a xenograft mouse model: CRABP-II-overexpressing cells gave rise to fewer tumors, and these were markedly smaller than tumors that developed from the parental cell-line (Table 1; Fig. 2). The ability of CRABP-II to suppress tumor growth was additionally tested in an immunocompetent mouse model that better mimics the gradual progression of human breast cancer. Adenoviral-mediated expression of CRABP-II in tumors that develop in female *MMTV-neu* mice led to a delay in tumor formation (Fig. 4) and, consequently, to a significant increase in overall survival rate (Fig. 5). Taken together, the observations demonstrate that CRABP-II markedly sensitizes mammary carcinomas to RA-induced growth inhibition both in cultured cells and in physiologically relevant models of breast cancer.

Interestingly, in both mouse models tested, no significant additional effects were observed on treatment of the animals with RA (Table 1; Figs. 2, 4, and 5). Thus, it appears that the RA-sensitizing effects of CRABP-II overexpression *in vivo* occur at endogenous hormone levels. Strikingly, administration of RA alone did not have beneficial effects in either of the mouse models. It is interesting to note that, although not statistically significant, RA treatment in the absence of CRABP-II overexpression resulted in a trend that suggested that high concentrations of the hormone may be detrimental (Table 1; Fig. 6). Indeed, it has been reported that RA may potentiate proliferation induced by epidermal growth factor in cultured explants of mouse mammary gland (43), inhibit lobuloalveolar differentiation of cultured mouse mammary glands (44), and under certain conditions, promote at late stages of malignant progression by stimulating the invasive

Fig. 6. Effects of CRABP-II expression and RA treatment on the characteristics of mammary carcinomas in *MMTV-neu* mice. Mice were killed 3 days and 1 h, respectively, after injection with adenovirus and with BrdUrd. Apoptotic cells (arrowheads) in mammary tumors treated either with AdCRABP-II (A) or Ad0 (B) were detected according to morphological criteria after staining with hematoxylin. Calibration bar, A and B, 20 μ m. The percentage (mean) of apoptotic (C) and BrdUrd-labeled (D) cells was determined for mice treated with RA (RA treatment +): Control ($n = 3$), AdCRABP-II ($n = 3$), Ad0 ($n = 2$), and for those without RA treatment (RA treatment -): Control ($n = 4$), AdCRABP-II ($n = 5$), Ad0 ($n = 6$) as described in "Materials and Methods." For the estimated apoptotic indices, two-tailed unpaired t test yielded P s of 0.0569 for AdCRABP-II+RA versus Ad0+RA, 0.012 for AdCRABP-II+RA versus Control+RA, 0.0002 for AdCRABP-II-RA versus Ad0-RA, and 0.0019 for AdCRABP-II-RA versus Control-RA. For the BrdUrd indices, $P = 0.0169$ for Control+RA versus CRABP-II+RA and 0.0315 for Control-RA versus CRABP-II-RA; bars, \pm SD.



capacity of some cell variants in the breast cancer tumor population (45). Taken together, the data indicate that CRABP-II is a critical component of the pathway that mediates the effects of RA on tumor formation in both the SC115 xenografts and the transgenic *MMTV-neu* models.

Expression of CRABP-II in the *MMTV-neu* mice suppressed tumor growth by induction of proapoptotic pathways and not by affecting cell cycle progression (Fig. 6). It is worth noting in regard to this that, in different models, retinoids were shown to inhibit carcinoma growth by a variety of mechanisms, including induction of differentiation (46), cell-cycle arrest (47), apoptosis (48, 49), or by a combination of effects (50, 51). One mechanism that has been repeatedly proposed to underlie RA actions in carcinomas is the modulation of growth-factor receptor expression. Specifically, it was proposed that these activities are mediated via down-regulation of the expression of Erb-B2/*neu* (52). It was reported in regard to this that the human *Erb-B2/neu* promoter is under RA regulation (53, 54) and that expression of the *neu* proto-oncogene decreases on RA treatment in a number of breast cancer cell lines (55, 56). The data presented here demonstrate a pronounced effect of retinoid signaling on tumor growth in the context of a transgenic *neu* construct, which is not under the regulation of the native *neu* promoter. Thus, other mechanisms that mediate the growth-inhibitory responses to RA must exist, and, in the model systems examined here, those appear to involve CRABP-II. Interestingly, it was reported recently that overexpression of *neu* is accompanied by RA resistance in some cells, and thus, a causal link between the two was put forward (42). It was additionally suggested that the RA responsiveness of these cells depends on the activity of Akt, a Ser/Thr kinase that is known to regulate survival pathways (42). Our observations that RA resistance in an elevated *neu* background can be reversed by expression of a specific RA binding protein raise new questions regarding the mechanisms by which the binding protein functions. It is usually believed that growth-inhibition by RA is mediated via the transcriptional activities of the hormone, and our previous data demonstrated direct cooperation between CRABP-II and the nuclear receptor RAR (22–24). Thus, molecular-level understanding of the functional link between signaling by RA and *neu* awaits additional investigation.

The data presented here is of clinical significance. In view of the RA resistance of various cancers, and the marked toxicity that accompanies pharmacological RA administration in human patients, our findings that CRABP-II can reverse RA resistance and that it does so without exogenous RA administration offer the possibility that targeted overexpression of this protein in early mammary tumors may be of potential benefit.

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