Cellular Retinoic Acid-Binding Protein(II) Presence in Rat Uterine Epithelial Cells Correlates with Their Synthesis of Retinoic Acid†

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ABSTRACT: Vitamin A (retinol) and retinoic acid are necessary for the maintenance of the female reproductive system of higher animals. Our previous work has demonstrated cell specific expression of cellular retinoic acid-binding protein (CRABP) and cellular retinoic-acid binding protein(II) [CRABP-(II)] in the uterus of the rat. CRABP(II) expression was shown to be induced in the uterine surface epithelial cells by treatment of prepubertal rats with pregnant mare serum gonadotropin (PMSG). Here we report that, after PMSG treatment, collected uteri had markedly higher levels of retinoic acid than did the uteri of prepubertal rats treated with the control vehicle. Smooth muscle, stromal, and epithelial cells were then cultured from uteri from such animals and provided with retinol or with the retinol/retinol-binding protein complex. Retinoic acid production, analyzed by high-performance liquid chromatography, was observed for the epithelial cells from the uteri of prepubertal animals treated with PMSG, cells previously shown to express CRABP(II) and confirmed here to continue to express it in culture. Little or no retinoic acid was produced by cultured epithelial cells from the prepubertal uteri [shown previously to be negative for CRABP(II)] or by smooth muscle and stromal cells taken from uteri of prepubertal or PMSG-treated rats (shown previously to express CRABP). Retinoic acid production by uterine epithelial cells [and CRABP(II) expression] was also observed if the prepubertal rat was treated with estrogen before cell collection. At no time did cells expressing CRABP exhibit significant retinoic acid synthesis. Thus, this system revealed an important difference in retinoid metabolism between cells expressing CRABP and CRABP(II) and suggests CRABP(II) may participate in retinoic acid production and/or secretion.

Retinoids, and retinoic acid in particular, are known to be necessary for the maintenance of many differentiated epithelial cells. Part of the cellular machinery that is involved in retinoid metabolism and function is a group of four intracellular retinoid-binding proteins. Two of these proteins bind retinol: cellular retinol-binding protein (CRBP)[1] and CRBP(II); the other two bind retinoic acid: cellular retinoic acid-binding protein (CRABP) and CRABP(II). The two pairs have significant sequence identity and binding specificity (Ong et al., 1994). The need for two closely related cellular retinol-binding proteins can be explained by their different locations and interactions with retinoid-metabolizing enzymes (Ong et al., 1994; Ong, 1994). Apo-CRBP in the liver competitively inhibits esterification of retinol to retinyl esters by lecithin−retinol acyltransferase (LRAT), while promoting hydrolysis of existing retinyl esters. The presence of apo-CRBP might serve as a signal that retinol intake is low and retinol should be mobilized from ester stores. CRBP(II), located in the enterocytes of the intestinal villi in considerable abundance, shows only a modest inhibitory effect on LRAT when it is in the apo form. Since formation of ester is required for export of retinol from the enterocyte, lack of LRAT inhibition allows proper retinoid processing when CRBP(II) might be in considerable stoichiometric excess over cellular retinol, depending on vitamin A intake.

In contrast to our understanding of the cellular retinol-binding protein pair, at this time, there is no clear understanding of why two closely related cellular retinoic acid-binding proteins are present in animals. It is known that retinoic acid mediates biological effects on target cells by binding to a family of retinoic acid receptors (RARS/RXRs) that interact with retinoic acid receptor specific sequences in promoter areas of target genes (Mangelsdorf et al., 1994). But despite the considerable amount of work demonstrating that retinoic acid regulates the expression of specific genes, there is still much to learn about which cells synthesize retinoic acid, and what roles the CRABPs might play in modulating retinoic acid action.

The uterus is an organ which is subject to retinoid action. The cellular retinoid-binding proteins CRBP and CRABP have been shown to be present in the human uterus (Chytil et al., 1975). The prepubertal and pseudopregnant rat uterus was recently examined for the presence of retinoid binding

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[1] Abbreviations: BSA, bovine serum albumin; CRBP, cellular retinol-binding protein; CRBP(II), cellular retinol-binding protein, type two; CRABP, cellular retinoic acid-binding protein; CRABP(II), cellular retinoic acid-binding protein, type two; hCG, human chorionogonadotropin; LC/ESI MS, liquid chromatography/electrospray ionization tandem mass spectroscopy; LRAT, lecithin−retinol acyltransferase; PMSG, pregnant mare serum gonadotropin; RBP, retinol-binding protein; ROL, retinol; RAR, retinoic acid receptor; RXR, retinoid X receptor.

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proteins (Bucco et al., 1996). CRBP and RBP are found in prepubertal stromal cells; CRABP is found in prepubertal and proliferative (induced by PMSG) stromal cells and smooth muscle cells at all stages and CRABP(II) in surface epithelial cells in the uterus of the PMSG-treated rat. Retinoic acid receptor α (RAR α) is found in epithelial, glandular, and stromal cells of the rat uterus (Zhuang et al., 1994). The presence of both CRABP and CRABP(II) in the uterus at particular times provides a system for investigating their potential roles. Here we determined that the uterus itself synthesized retinoic acid. Retinoic acid synthesis was observed for cultured uterine epithelial cells but only at the time when CRABP(II) was expressed. Retinoic acid synthesis was not observed for the stromal or smooth muscle cells that expressed CRABP. CRABP(II) thus was a marker for cells actively synthesizing retinoic acid and may participate in that process.

EXPERIMENTAL PROCEDURES

Animals and Hormones. Female Sprague−Dawley rats (19 days old on delivery) were housed in a temperature- and light-controlled room (21 ± 1 °C, lights on from 7:00 a.m. to 7:00 p.m.). Rats were fed rat chow (Ralston-Purina Co., St. Louis, MO), provided with water *ad libitum*, and allowed to acclimate for 6 days before use in these experiments. Studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the oversight of veterinarians and our local Institutional Animal Care and Use Committee.

PMSG was purchased from Sigma Chemical Co. (St. Louis, MO; lot 39F-02481; 4150 IU/mg). Human CG (CR125; 11 900 IU/mg) was a gift from the Center for Population Research, NICHD/NIH (Bethesda, MD).

Hormone Treatments. Induction of pseudopregnancy was as previously described (Bucco et al., 1996). Briefly, female rats (24 days old) received either a 20 IU injection of PMSG to induce follicular growth and development or a control injection (saline). Two days later (26 days old), the control animals ($n = 15$) and some of the PMSG-treated rats ($n = 15$) were killed and their uteri were removed and separated from connective tissue, fat, and the oviduct. Uteri were then snap frozen on dry ice until subsequent retinoic acid extraction and HPLC analysis (see below). The remaining PMSG-primed rats ($n = 15$) received an ovulatory dose of hCG (10 IU), and their uteri were collected 24 h later for retinoic acid analysis. Estrogen pellets were placed in animals as previously described (Bucco et al., 1996) to assess the ability to induce retinoic acid production in uterine epithelial cells.

Primary Uterine Cell Cultures. The isolation of uterine epithelial, stromal, and muscle cells for primary cell culture was accomplished as previously described (Osteen et al., 1989) with several modifications. Uteri ($n = 15$) were slit longitudinally and placed in medium containing 0.4% collagenase, 0.02% DNAse, and 2% chicken serum for 30 min at 4 °C. This was followed by incubation at 37 °C for 1 h. The uteri were vortexed at medium speed for 30 seconds (six times). The supernatant liquid containing stromal and epithelial glands was collected. The stromal cells were isolated from epithelial fragments by sequential filtration through 88 and 20 μm filter units. The myometrial cells were prepared by washing the remaining uterine material with Hank's balanced salts, followed by incubation in 0.05% trypsin and 0.02% DNAse at 37 °C for 30 min. The digestion was stopped by the addition of 2% calf serum. The cells were washed in medium with DNAse, and the cell pellet was resuspended in serum free medium and passed through a 20 μm filter. The retentate was discarded and the effluent, which contained the muscle cells, collected. The cells were washed, counted, and plated on type I rat tail collagen.

The cell viability was evaluated by trypan blue dye exclusion (>95%). The purity of epithelial and stromal cell populations was defined by the histochemical marker Cytokeratin PKK-1 (Labsystems, Helsinki, Finland), a mouse monoclonal antibody which cross reacts with rat cytokeratins that are found in uterine epithelial cells but not stromal cells. Muscle cell populations were judged visually by morphology. All cell populations were found to be >96% pure. CRABP and CRABP(II) expression in cells was confirmed by immunohistochemistry as described in detail elsewhere (Bucco et al., 1996).

Cell populations were isolated and plated by midafternoon, allowed to grow for 36 h, and then provided with a control vehicle (PBS), 2.0 mM human retinol-binding protein purified from plasma (Davis & Ong, 1992), or 8.0 mM bovine serum albumin followed by 2.0 mM retinol. Incubations with BSA/retinol ended at 6 h, while groups provided with the control vehicle and RBP ended after 24 h.

Determination of Retinoic Acid. The procedures used for retinoic acid analysis have been described in detail (Napoli, 1986; Pappas et al., 1993) and followed here with the following exception. Pooled hexane fractions containing retinoic acid were first passed over a column of 0.1 g of 70−230 mesh silica (Aldrich, St. Louis, MO) in order to remove material that degraded resolution.

Media from two wells of a 24-well cell culture plate containing 1 mL of Dulbecco's Modified Eagle's Media and 300 000 cells (either smooth muscle, stromal, or epithelial) were added to 6 mL of 100% ethanol to start the extraction procedure in the primary cell culture experiments. HPLC separations were accomplished with an ISCO (Lincoln, NE) model 2350 pump and model 2360 gradient programmer. Retinoic acid was separated on a Whatman Partisil 5 (4.6 mm × 25 cm) 5 mm particle silica column with a Supelco LC-Si 2 cm guard column (Bellefonte, PA). The mobile phase was 92:8:0.1 hexane/dioxane/acetic acid at 2 mL/min. A V^4 absorbance detector set at 352 nm with a Spectra-Physics SP4270 (San Jose, CA) integrator was used for retinoic acid detection. Under the methods employed here, no detectable retinoic acid was produced from control tissue and culture extractions containing exogenous retinol added in excess of retinol levels employed in the experimental regimen. Production of 13-*cis*-retinoic acid from *all-trans*-retinoic acid did occur in control extractions, the amount increasing with the time taken to dry samples under nitrogen. Thus, the 13-*cis*-retinoic acid peaks observed were considered to arise from *all-trans*-retinoic acid. Retinoic acid levels were corrected for recovery by the addition of known amounts of *all-trans*-retinoic acid to mock sample groups during analysis.

HPLC/ESI MS and MS/MS Analysis of all-trans-Retinoic Acid. LC/ESI MS (liquid chromatography/electrospray ionization tandem mass spectroscopy) and MS/MS studies were carried out on a Finnigan TSQ-7000 instrument

interfaced with an HP 1090 series II liquid chromatograph. Solvent A was 5 mM aqueous ammonium acetate containing 0.04% acetic acid; solvent B was 5 mM methanolic ammonium acetate containing 0.04% acetic acid. A solvent composition of A/B (1:1) at a flow rate of 200 μL/min was used. Nitrogen gas (Liquid Air, Walnut Creek, CA) was used as a sheath (70 psi) and auxiliary (20 psi) gas to assist with nebulization. An ESI spray voltage of 4.5 kV was applied to the spray needle. The capillary was heated to 200 °C in order to provide optimal desolvation. The ES interface and mass spectrometer parameters were optimized to obtain maximum sensitivity without sacrificing the unit resolution. Argon (Liquid Air) was used as a collision gas. The collision cell pressure and skimmer pump pressure were 2.22×10^{-3} and 8.47×10^{-1} Torr, respectively.

RESULTS

Rat Uterine Tissue Retinoic Acid Content. We have previously reported that CRABP(II) expression is induced in the surface epithelium of the uterus of prepubertal rats treated with PMSG and its expression is then abrogated by subsequent treatment with hCG (Bucco et al., 1996). Since CRABP(II) mRNA and protein levels have been shown to be increased by the addition of *all-trans*-retinoic acid in other systems [e.g. Elder et al. (1993)], we examined here the possibility of whether retinoic acid might be found in greater abundance in the uterus at the time when CRABP(II) was expressed. Analyses for retinoic acid were carried out on uteri collected from prepubertal (Figure 1a) and PMSG [48 h (Figure 1b)]- and hCG [24 h (Figure 1c)]-treated rats. Uteri from the PMSG-treated animals, containing CRABP(II), consistently demonstrated the highest levels of retinoic acid, while no retinoic acid was detected in the uteri from animals 24 h post-hCG treatment, when CRABP(II) expression has ceased. Little retinoic acid was recovered from the uteri of the prepubertal animals. Thus, expression of CRABP(II) correlated with the presence of endogenous retinoic acid. While this pattern remained consistent in subsequent experiments, the amount of recovered retinoic acid varied, which may have been due to animal variation and/or inexact dating prior to shipping.

Retinoic Acid Production by Primary Uterine Cell Culture. The coincidence of the presence of retinoic acid and the appearance of CRABP(II) suggested the possibility that retinoic acid might be synthesized within the uterus itself. To test this idea, primary cell cultures of rat uterine smooth muscle, stromal, and epithelial cells were examined for the ability to synthesize retinoic acid when provided with retinol. Cell cultures were incubated with either 8 μM BSA/2 μM ROL or 2 μM human RBP/ROL (>95% saturated). Both methods of retinol delivery were employed in order to ensure that any differences in synthesis would not be due to altered expression of RBP receptor (Bavik et al., 1995). Retinol with BSA allows nonspecific entry into cells.

Little or no retinoic acid was produced by the cells isolated from prepubertal control animals (Figure 2), consistent with the failure to detect significant quantities of endogenous retinoic acid in the whole uterus. After induction of prepubertal animals with PMSG, the collected uterine epithelial cells, which were now expressing CRABP(II), demonstrated a potent ability to synthesize retinoic acid when provided with 2 μM human RBP/ROL (Figure 3c). In

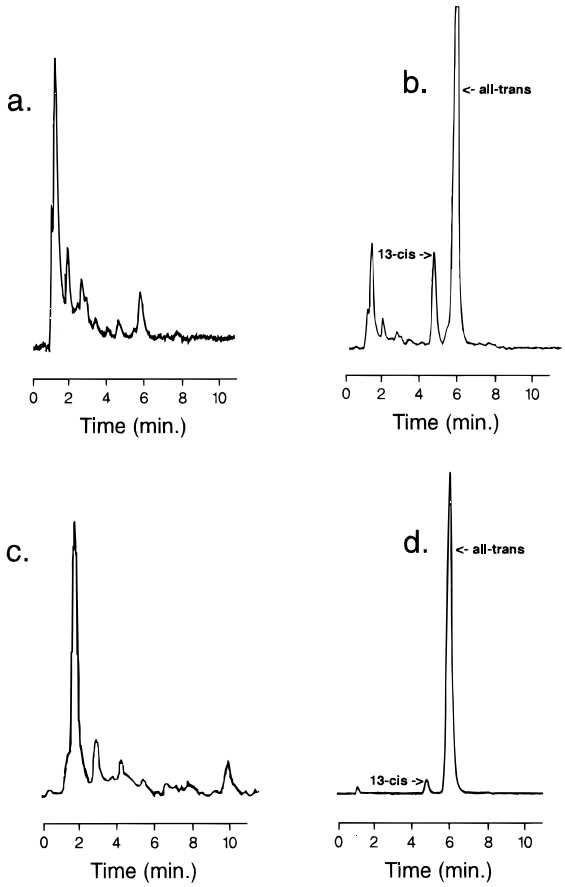

FIGURE 1: Retinoic acid levels in whole uteri taken from PMSG/hCG-treated rats. HPLC chromatograms showed small quantities of *all-trans*-retinoic acid in prepubertal control rats (a), which are dramatically increased by 48 h post-PMSG treatment (b), falling to nondetectable levels in PMSG/hCG (24 h) uteri (c). *all-trans*- and 13-*cis*-retinoic acid peaks derived from samples were at the appropriate retention time established with standards (d). The data shown are representative of half-sample injections of whole uteri retinoic acid extractions ($n = 4$ uteri for each sample). Tracings were at 0.005 AUFS with attenuation in panels a−c, adjusted to 0.25 of that in panel d.

contrast, both the stromal and the smooth muscle cells, both of which express CRABP, had little if any ability to synthesize retinoic acid. The small peak seen for the stromal cells at >7 min was not at the retention time seen for retinoic acid. Given the limits of detection (0.5 pmol), we can estimate that the amount of retinoic acid produced was at least 100-fold greater for the epithelial cells after PMSG, compared to any other cell preparation. The presence of CRABP(II) and CRABP in the cultured uterine cells was confirmed by immunohistochemical analysis (data not shown), in agreement with our previous work demonstrating binding protein expression in these cells in the intact organ (Bucco et al., 1996). Synthesis of retinoic acid by the epithelial cell was unexpected. One might have predicted that the retinoic acid would be produced by other cells to act on the epithelial cells, dependent on vitamin A for proper differentiation.

In our hands, the pattern of retinoic acid production was the same for all subsequent experiments whether either 8 μM BSA/2 μM ROL or 2 μM human RBP/ROL was used. The 8 μM BSA/2 μM ROL treatments clearly demonstrated higher levels of retinoic acid production by uterine epithelial cells than did the 2 μM human RBP/ROL treatments (see Table 1), which is consistent with previous work demonstrating greater acylation of retinol by cellular LRAT when BSA/

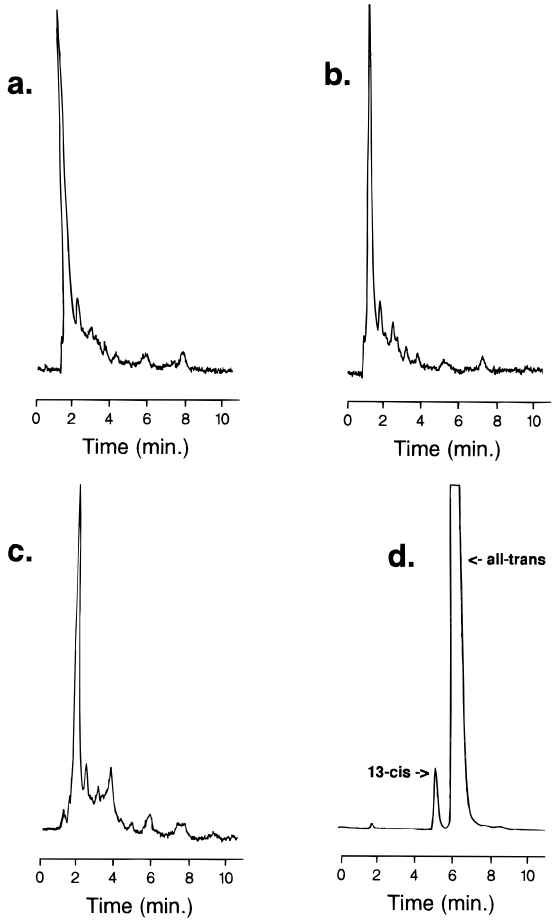

FIGURE 2: Retinoic acid production by uterine cells from the prepubertal rat, cultured with RBP/ROL. Little or no retinoic acid was detected when prepubertal uterine smooth muscle (a), stromal (b), and epithelial (c) cells were cultured with 2 μM RBP/ROL. The elution position of authentic 13-*cis*- and *all-trans*-retinoic acid is shown in panel d. The data shown represent full sample injections of extractions from the media of four wells (1.0 mL each) from a 24-well cell culture dish containing 3×10^5 cells in each well. Tracings were at 0.005 AUFS with attenuation in panels a−c, adjusted to 0.25 of that in panel d. As little as 0.5 pmol of retinoic acid produced a clear peak in this system.

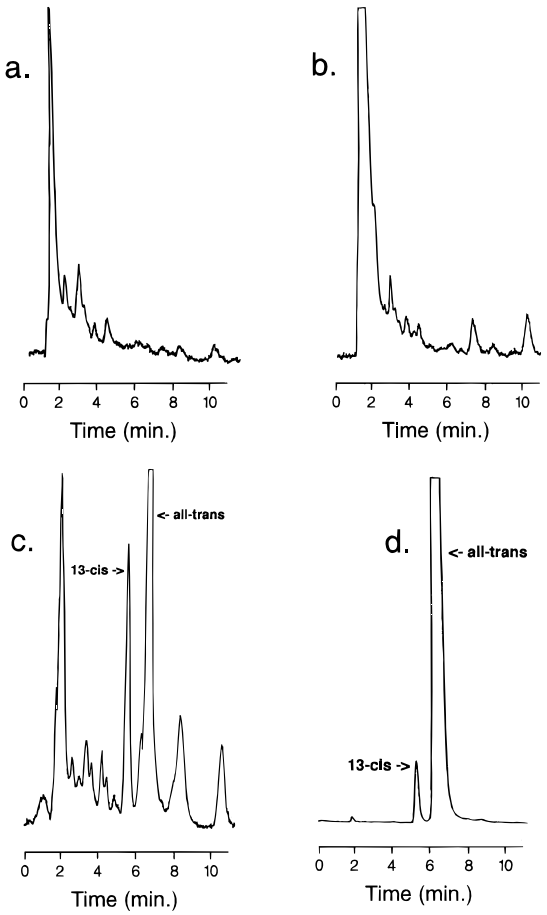

FIGURE 3: Retinoic acid production by uterine cells obtained from the PMSG-treated rat, cultured with 2 μM RBP/ROL. Little or no retinoic acid was detected in the culture of smooth muscle cells (a). Small peaks were detected for extracts of cultures of stromal cells but were not clearly identifiable as retinoic acid (b). Larger peaks of retinoic acid (equivalent to 40 pmol) were detected in the cultures of uterine epithelial cells (c), compared to the elution profile of authentic 13-*cis* and *all-trans*-retinoic acid standards (d). The data shown represent full sample injections of extractions containing the media of four wells (1.0 mL each) from a 24−well cell culture dish containing 3×10^5 cells in each well. Tracings were at 0.005 AUFS with attenuation in panels a−c, adjusted to 0.25 of that in panel d. As little as 0.5 pmol of retinoic acid produced a clear peak in this system.

retinol was the carrier provided versus RBP/retinol (Shingleton et al., 1989). This difference is probably due to increased substrate availability of the retinol precursor because more retinol enters the cell nonspecifically when it is not bound to RBP. The BSA was included in response to retinol extraction controls which showed greater stability of retinol when presented with BSA as a carrier compared to no carrier (data not shown).

Spectroscopic Confirmation of all-trans-Retinoic Acid Synthesis by Uterine Epithelial Cells. To confirm the identify of the material eluting at the retention time established for retinoic acid, midpeak duplicate fractions were combined and analyzed by absorbance spectroscopy (Figure 4a). The absorbance maximum occurred at ~350 nm, which was considered indistinguishable from the ~352 nm maximum obtained for authentic *all-trans*-retinoic acid in acidic ethanol. The shape of the spectrum was the same as that of authentic retinoic acid. Addition of base to the recovered material shifted the spectral maximum to ~335 nm, just as observed for authentic retinoic acid anion (data not shown).

To confirm further the chemical structure of the biosynthetic product that comigrated with the *all-trans*-retinoic acid standard on HPLC, LC/ESI MS/MS analysis was performed. The ESI spectrum of the product showed a MH^+ ion at *m/z* 301 (Figure 4b). For further elucidation of the structure, product ion spectra were obtained for precursor ions at *m/z* 301 (data not shown). The LC MS/MS spectrum of *m/z* 301

Table 1: Synthesis of *all-trans*-RA by Rat Uterine Cells

cell type (treatment)	BSA/ROL[a] [pmol of RA/(10^6 cells)]	RBP/ROL[b] [pmol of RA/(10^6 cells)]
epithelium (control)	8.2 ± 3.4[d]	nd[c]
epithelium (PMSG)	210 ± 25[d]	36 ± 8[d]
epithelium (estrogen)	142 ± 5[e]	21 ± 4[f]
stroma (control)	nd	nd
stroma (PMSG)	7.4 ± 2.8[d]	nd
smooth muscle (control)	nd	nd
smooth muscle (PMSG)	nd	nd

[a] 6 h treatment time (8 μM BSA/2 μM ROL). [b] 24 h treatment time (2 μM RBP/ROL). [c] nd = not detectable. [d] Average of four determinations from a total of 30 rats. [e] Average of two determinations from a total of 15 rats. [f] Average of three determinations from a total of 15 rats.

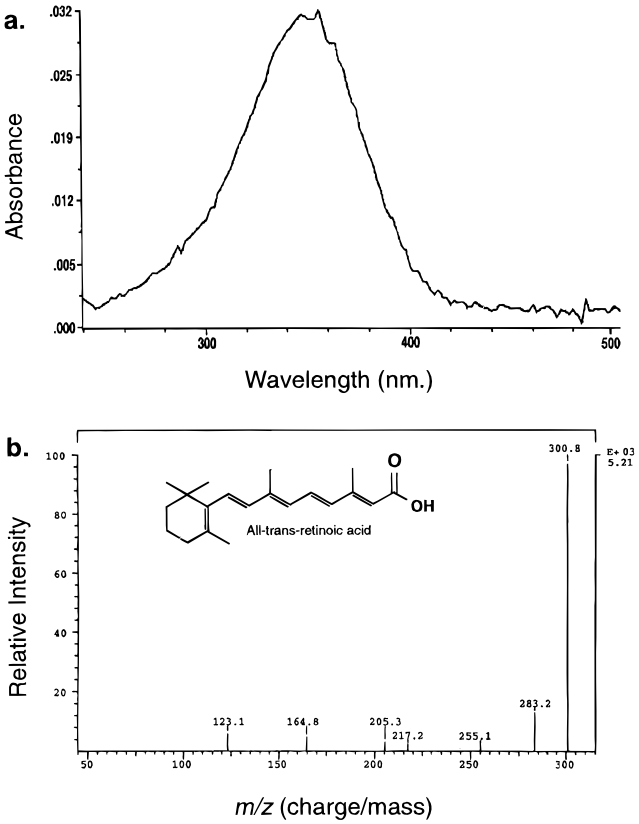

FIGURE 4: Spectroscopic analysis of *all-trans*-retinoic acid peaks recovered after HPLC. Absorbance spectroscopy (a) of the peak (obtained from duplicate extractions of epithelial cells and culture medium) eluting at the retention time established for *all-trans*-retinoic acid showed a peak absorbance at ~350 nm (λ_{max} for RA = 352 in acidic ethanol). The spectrometer was blanked prior to reading with the HPLC mobile phase (92:08:0.1 hexane/dioxane/acetic acid). These fractions were dried under nitrogen in the dark and submitted for mass spectroscopic analysis (b), which showed a parent ion molecular weight equal to that for retinoic acid.

at a −15 eV collision offset showed diagnostic fragment ions at *m/z* 179 and 123 (resulting from the cleavage of the aliphatic side chain), 283 ($MH^+ - H_2O$), and 205 ($MH^+ - CHCH(CH)_3CHCOOH$). A similar mass spectral profile was obtained with authentic *all-trans*-retinoic acid.

Induction of Retinoic Acid Production by Estrogen in Uterine Epithelial Cells. CRABP(II) mRNA had been found to be induced in rat uterine epithelial cells within 4 h of estrogen treatment (Bucco et al., 1996). We examined the possibility that estrogen treatments that induced CRABP(II) appearance might also induce retinoic acid production. Epithelial cells from uteri of estrogen-treated animals (12 h) produced considerably more *all-trans*-retinoic acid than did untreated prepubertal controls, comparable to that seen for PMSG-treated animals (Table 1). CRABP(II) expression was made evident by immunohistochemical analysis in the epithelial cells taken from estrogen-induced animals (data not shown), in agreement with previous work done with the intact animal (Bucco et al., 1996).

Although retinoic acid production from uterine epithelial cells was clearly demonstrated after treating 24-day-old rats with estrogen pellets, placing naive prepubertal control epithelial cells in an estrogen environment (1×10^{-8} M) did not induce retinoic acid production. It is probable that, if estrogen acted directly as a stimulus for retinoic acid production in the intact animal, other growth factors are also needed in this process, which were not present in the medium for the cells cultured from the prepubertal animal. This would be consistent with the network of complex stromal−epithelial interactions reported in the uterus (Cuhna et al., 1985).

Previously, we had observed that uterine surface epithelial cells, but *not* glandular epithelial cells, expressed CRABP(II) after PMSG or estrogen treatments of prepubertal rats. We separated glandular epithelium from surface epithelium by washing cells through an additional 20 μm filter, with surface epithelial cells passing through and glandular epithelial cells being retained. Incubation of these two epithelial cell populations with 8 μM BSA/2 μM ROL resulted in virtually all retinoic acid production being confined to the single cell surface epithelial cell populations; any retinoic acid production by glandular epithelial cells was below detectable levels. Similarly, surface epithelial cells isolated from the uteri of animals post-hCG, when CRABP(II) expression had ceased (Bucco et al., 1996), did not synthesize retinoic acid, consistent with the lack of retinoic acid in the uterus demonstrated for these animals in the first experiment. Thus, in the systems examined here, CRABP(II) was a marker for cells that were capable of retinoic acid synthesis.

DISCUSSION

One postulate has been that CRABP and CRABP(II) might fulfill similar roles, restricting retinoic acid from reaching the nuclear receptors. This would allow certain cells to "ignore" the presence of retinoic acid while other cells in the same environment, not expressing either binding protein, could respond. In addition, expression of one of the binding proteins might also allow paths of proliferation or differentiation to proceed that would otherwise be blocked by retinoic acid action [e.g. Bucco et al. (1995)]. Both binding proteins can been seen to be excluded from the nucleus in certain cells in which they are expressed (Bucco et al., 1995; Zheng et al., 1996), consistent with a protective effect. The need for two retinoic acid binding proteins might then be explained by the fact that it would allow different signals to regulate expression of the "retinoid block".

The results presented here could be considered consistent with that hypothesis but with the added point that CRABP(II) is employed to protect cells that are actively synthesizing retinoic acid for export while CRABP is found in cells that are exposed to that exported retinoic acid but that are not the target of that signal. Further, the role of CRABP(II) might not be simply protective; it might participate in the process of synthesis and/or release as well. The ability to be induced by retinoic acid would fit the needs of the system examined here, where the epithelial cells only produce retinoic acid under certain stimuli. The expression of CRABP(II) can then be regulated by the induction of the retinoic acid-synthesizing enzymes. In the normally cycling rat, CRABP(II) expression is restricted to the epithelial cells during estrous (Wardlaw et al., 1997).

That the enzyme system is induced can be postulated from the observation that epithelial cells from the uterus of prepubertal or post-hCG-treated animals did not synthesize retinoic acid even when given retinol that would enter the cell nonspecifically. Whether CRABP(II) is induced only by retinoic acid or might also be responsive to the same

signal(s) that induces retinoic acid synthesis needs to be examined further.

Since little or no retinoic acid was synthesized by either uterine smooth muscle or stromal cells, and virtually all cells isolated in the epithelial cell fraction stained positively for the epithelial cytokeratin PKK-1, the purity of epithelial cell populations and the specificity of retinoic acid production from these cells should be regarded with confidence. Clearly, CRABP was not associated with significant retinoic acid production.

Ovarian granulosa cells also express CRABP(II) after PMSG treatment (Bucco et al., 1995), and preliminary studies indicate that these cells also produce retinoic acid from the same precursors employed in this work. Thus, the coincidence of CRABP(II) expression and retinoic acid synthesis may be a general phenomenon. If this holds true, then patterns of CRABP(II) expression during embryogenesis might be important clues to local sites of retinoic acid production. However, it is clear that retinoic acid production does not require the presence of CRABP(II), because cells not known to contain the protein have been demonstrated to synthesize retinoic acid in vitro. However, it may be that the presence of CRABP(II) indicates the production of retinoic acid synthesis in some systems, perhaps those that only produce retinoic acid at particular times rather than constituitively. That CRABP(II) is not necessary for retinoic acid synthesis even in those cells that normally express it is clear from the relatively normal phenotype observed for the CRABP(II) knockout mouse (Fawcett et al., 1995; Lambron et al., 1995).

There has been little work done in identifying cell types that synthesize retinoic acid. Synthesis of retinoic acid from cultured rabbit Muller cells has been reported, but whether those cells express CRABP(II) has not been established (Edwards et al., 1992). To our knowledge, this work represents the first demonstration of retinoic acid production stimulated by the steroid hormone estrogen. Boettger-Tong and Stancel (1995) recently showed that retinoic acid inhibits estrogen-induced uterine stromal and myometrial cell proliferation. A hypothesis consistent with the current data would be that the estrogen produced by the ovary during the proliferative phase of the uterine cycle initiates cell proliferation and that subsequent retinoic acid production by uterine epithelial cells promotes differentiation of stromal and myometrial cells. Thus, retinoic acid would block further proliferation by uterine cells and prepare the uterus for imminent implantation.

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