

# Retinoic Acid Biosynthesis by Normal Human Breast Epithelium Is via Aldehyde Dehydrogenase 6, Absent in MCF-7 Cells<sup>1</sup>

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## ABSTRACT

Retinoic acid (RA) is the form of vitamin A that controls differentiation and proliferation of epithelia. Our previous work established that normal breast epithelia synthesize RA from retinol, an ability retained by three immortalized but nontumorigenic cell lines but lost in five of six breast cell lines. In this work, we characterize the cause of this defect in one of the lines, the MCF-7 line. We have determined that the immortalized but nontumorigenic cell line, MTSV1.7, capable of synthesizing RA from both retinol and retinal, contains a retinaldehyde dehydrogenase activity for the second step in RA biosynthesis. We have identified it, after isolation, as a previously described enzyme, aldehyde dehydrogenase 6 (ALDH6). Immunohistochemical analysis of normal human breast with antibodies to ALDH6 showed expression of this enzyme in the glandular epithelia colocalized with cellular RA-binding protein type II, a possible marker for certain cells able to synthesize RA. ALDH6 was not present in MCF-7 cells, and these cells were unable to oxidize retinal to RA in culture. When MCF-7 cells were then transfected with ALDH6, they (re)gained the ability to oxidize retinal to RA as well as some ability to synthesize RA when provided with retinol. This suggests that loss of ALDH6 expression is the defect in RA biosynthesis in these cells. Identification of ALDH6 as the retinaldehyde dehydrogenase present in normal human breast epithelia provides the first tool necessary for studying the loss of RA synthetic ability in cancer cells and the relationship of this process to malignant transformation.

## INTRODUCTION

An important biological activity of vitamin A is the control of the differentiation and proliferation of various epithelia of the body. The studies of Wolbach and Howe (1) in 1925 demonstrated that the absence of vitamin A leads to the uncontrolled proliferation of epithelial stem cells that fail to differentiate to the normal phenotype in many lining epithelia. Histological changes in vitamin A-deficient rats showed both the abnormal differentiation of epithelia to a squamous, keratinizing phenotype and the hyperproliferation of this abnormal epithelium (1). Interestingly, the histological changes they observed at some sites included invasion of other cellular layers by the proliferating cells with incorporation of new vasculature, leading them to observe that this was “growth power of neoplastic potentiality.” The ability of vitamin A acid, RA,<sup>3</sup> to prevent or reverse such changes (2, 3) has prompted numerous investigations of the possible involvement of members of the vitamin A family, the retinoids, in the development or prevention of certain cancers. Some of these efforts have been directed toward identifying changes in the cellular components important for retinoid action, beginning 25 years ago (4).

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<sup>3</sup> The abbreviations used are: RA, retinoic acid; RAR, retinoic acid receptor; ALDH, aldehyde dehydrogenase; CRABP(II), cellular retinoic acid-binding protein, type II; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; HPLC, high-performance liquid chromatography; RALDH, retinaldehyde dehydrogenase;  $\beta$ ME, 2-mercaptoethanol.

All-*trans*-RA activates and represses gene transcription through binding to the retinoid nuclear receptors of the RAR family (5). An important part of this process, then, is the generation of RA from retinol. A number of studies have identified many possible biochemical activities for catalyzing the two-step oxidation of retinol to RA, which are as diverse as microsomal short-chain alcohol dehydrogenases, cytosolic alcohol and ALDHs, and cytochrome P-450 isozymes (Refs. 6–9 and references therein). What remains unclear, however, is exactly which of these activities is functioning at sites of RA synthesis in the adult. We have observed that one of the intracellular binding proteins for RA, CRABP(II), appears to be a marker for cells that synthesize RA at certain sites in the adult rat (10–12). This protein is expressed in normal human mammary epithelium, and when these epithelial cells are cultured, they will synthesize RA from retinol. However, five of six human breast cancer cell lines that we examined had little or no ability to carry out this synthesis, whereas three immortalized, nontransformed lines had retained the ability (13). This opened the possibility that a defect in RA synthesis might be involved in the process of malignant transformation. For this to be tenable, however, it must be confirmed that this defect exists in malignant or premalignant lesions *in situ*. This will require knowledge of the enzymology involved so that tools can be developed for such studies.

To initiate that process, we sought to characterize the biochemical defect preventing RA synthesis from retinol for one of the lines, the well-studied MCF-7 cell line. We compared these cells to an immortalized but nontumorigenic breast cell line that is competent in RA synthesis, MTSV1.7. Here we report that ALDH6 (14) is responsible for the second step of RA synthesis, oxidation of retinal to RA, in the MTSV1.7 cells and in normal mammary epithelia, and that expression of this enzyme and the ability to oxidize retinal to RA is lost in the MCF-7 cell line. This provides us with the first of the several tools that will be required to assess the importance of this process in the development of human mammary cancers.

## MATERIALS AND METHODS

**Cell Culture.** MTSV1.7 cells were a gracious gift of Dr. Joyce Taylor-Papadimitriou (Imperial Cancer Research Fund, London, United Kingdom) and were cultured as recommended in DMEM/F12 50:50 mix supplemented with 10% FBS and 0.5  $\mu$ g/ml hydrocortisone. MCF-7 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in DMEM/F12 50:50 mix, 10% FBS, and 0.5  $\mu$ g/ml insulin. NMU cells from American Type Culture Collection, derived from a rat mammary adenocarcinoma, were cultured in DMEM/F12 50:50 mix with 10% FBS.

To measure RA synthesis in culture, cells were seeded in 60-mm dishes and grown until just confluent. Normal medium (described above) was removed and replaced with 3 ml of medium containing 10  $\mu$ M BSA and 2  $\mu$ M retinol or retinal (provided in DMSO). Cells were incubated with retinoids for 4 h, then the cells were scraped into the medium, and cells and medium were extracted together. For quantitation, a duplicate plate of cells was trypsinized, pelleted, and lysed by sonication, and the protein amount was determined by BCA Assay kit (Pierce, Rockford, IL).

Transient transfection of human ALDH6 into MCF-7 cells was performed using SuperFect from Qiagen (Valencia, CA), following the manufacturer's protocol with slight modification. Cells were seeded at ~50% confluence in 60-mm dishes, and 5  $\mu$ g of DNA were complexed for 10 min at room temperature with 30  $\mu$ l of SuperFect in 150  $\mu$ l of serum-free medium. One ml

of normal medium was added to the complex and applied to cells for 3 h, after which complexes were left on cells and 3 additional ml of normal medium were added to the cells. Cells were assayed 36 h after transfection. Cells were transfected with the human ALDH6 sequence cloned into pcDNA 3.1(-) (Invitrogen, Carlsbad, CA) or vector alone as control.

**RT-PCR of RALDHs.** To investigate possible expression of known ALDHs, RT-PCR was performed with oligo(dT)-primed cDNA from MTSV1.7 cell RNA, using a general forward primer designed to a highly conserved region of the ALDH family, 5'-TTTGCAGATGCTGACTTGGAC, or specific forward primers for RALDHs 1 and 2, 5'-TGAGAGTGGGAA-GAAA-3' and 5'-CTTTGATCCCACTACTGAG-3', respectively. Forward primers were used in conjunction with a common reverse primer, 5'-CCATT-TCCAGACATCTTGA-3'.

**Isolation and Identification of ALDH6 in MTSV1.7 Cells.** MTSV1.7 cells were grown in 150-mm culture dishes, trypsinized, pelleted, and stored at -70°C before use. Cells from 20 150-mm plates were harvested, and the pellets were resuspended in 16 ml of buffer A [0.02 M Tris acetate (pH 8.3), 5 mM DTT] and sonicated three times for 30 s. After centrifugation at 170,000 × *g* for 30 min in a Beckman Optima TL Ultracentrifuge, the supernatant was applied to an HPLC ion-exchange column (LKB TSK DEAE-5PW column, 8 mm × 7.5 cm) equilibrated in buffer A at 1 ml/min. After one column volume of wash with buffer A, a gradient elution was started from 100% buffer A to 100% buffer B [0.2 M Tris acetate (pH 8.3), 5 mM DTT], over 20 ml. One-ml fractions were collected. Elution of protein was followed by monitoring absorbance at 280 nm with an Isco V<sup>4</sup> detector; the elution fraction of ALDH6 was identified by enzyme assay for RALDH activity, and the appropriate fractions were combined and concentrated to ~2-ml volume. This sample was then applied to an HPLC sizing column (TosoHaas TSKGel 300 SW; 20 mm × 30 cm) equilibrated with 0.1 M Na<sub>2</sub>SO<sub>4</sub> in 0.1 M sodium phosphate (pH 6.8). The column was run at 4 ml/min, and 2-ml fractions were collected. Previous experiments had identified the elution position of RALDH activity between fractions 29 and 33. These fractions were run on an 8% SDS-PAGE gel and stained with Coomassie Blue. Fractions were also assayed for their RALDH activity. A *M<sub>r</sub>* ~56,000 band, the intensity of which paralleled the measured RALDH activity, was cut from the gel and sent to Vanderbilt's Protein Chemistry Laboratory for tryptic digestion and mass spectrometric analysis of the peptides produced.

**RA Extraction and HPLC Analysis.** The procedures used for extraction and analysis of RA have been described previously (10) in detail. Briefly, cell culture medium and cells or *in vitro* reactions were mixed with an equal volume of 4.25 M NaCl, 0.25 M KOH, and 2× volume of 100% ethanol with 250 μg/ml butylated hydroxytoluene. Neutral retinoids were extracted twice with a volume of hexane + 250 μg/ml butylated hydroxytoluene equal to the aqueous phase and discarded. The aqueous phase was then acidified with 6 N HCl (0.03× volume), and acidic retinoids were extracted once with an equal volume of hexane. Extracted fractions were dried under nitrogen and dissolved in mobile phase (hexane:dioxane:acetic acid, 92:8:0.1) for HPLC separation. Retinoids were separated on a Whatman Partisil 5 silica column (4.6 mm × 250 mm) running at 2 ml/min. Detection was with a Waters 996 Photodiode Array detector and Millennium<sup>32</sup> software. RA was monitored at 352 nm. Quantitation was performed by relating the area of the peak to areas obtained by the analysis of known quantities of retinoid standards.

**In Vitro Assay of RALDH Activity.** Cells to be assayed were trypsinized and pelleted, and the pellets were resuspended in 4× volume of 20 mM HEPES (pH 7.4), 150 mM KCl, 2 mM EDTA, and 1 mM βME. Cells were sonicated with 10 1-s pulses, and cytosol was obtained by centrifugation in a Beckman TL-100 rotor at 170,000 × *g* for 30 min. Cytosolic protein was quantified by BCA Assay. Reactions were 0.2 ml in volume, with typically 100 μg of cytosolic protein, 100 μM NAD<sup>+</sup>, and 2 μM retinal (from a stock in DMSO) with or without 2 μM recombinant CRBP, produced as described previously (15). After incubating 20 min at 37°C, reactions were stopped by addition of ethanol and extracted as described above.

**Preparation of Recombinant ALDH6.** The open reading frames of human and rat ALDH6 were obtained by RT-PCR with oligo(dT)-primed RNA from MTSV1.7 cells or a rat mammary carcinoma cell line, NMU, as template. PCR was performed with forward primer 5'-GGGCCCCATATG(CAT)<sub>6</sub>GG-

CATGGCCACCGCTAACGGGGCC-3', containing a hexahistidine tag, and reverse primer 5'-GGGCCAAGCTTGCCTTTCCTTCAGGGTTCTT-3'. PCR products were gel purified, digested with *Nde*I and *Hind*III, and ligated into pT7-7 vector for bacterial expression. The insert containing the human ALDH6 open reading frame was removed from the pT7-7 vector by digestion with *Xba*I and *Hind*III and ligated into pcDNA 3.1(-) to construct a mammalian expression vector.

To produce recombinant ALDH6 protein, BL21-Gold(DE3)pLysS cells (Stratagene, La Jolla, CA) were transformed with the rat ALDH6-pT7-7 construct. A 50-ml culture was induced overnight at room temperature with 1 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation and lysed by sonication in 50 mM sodium phosphate, 250 mM NaCl, and 2 mM βME (pH 7.5). The 100,000 × *g* supernatant was incubated with 4 ml of a 50% slurry of nickel-nitrilo-triacetic acid resin (Qiagen) and then loaded into a column for washing with wash buffer [40 mM imidazole acetate, 1 mM *n*-octyl glucoside, and 10% glycerol (pH 8.0)]. The recombinant protein was eluted from the column with 200 mM imidazole acetate, 2 mM βME, 250 mM NaCl, 1 mM *n*-octyl glucoside, and 10% glycerol (pH 8.0). Purity of recombinant protein was assessed by visualization of only a single band by Coomassie Blue staining.

**Antibodies, Western Blots, and Immunohistochemistry.** A peptide sequence of ALDH6 with significant difference from RALDH-1 and RALDH-2 was chosen for immunization. The peptide, CCGSAMEDKGLFIKP, was synthesized by Peptidogenics (Livermore, CA). Two mg were conjugated to keyhole limpet hemocyanin with the Pierce Inject Maleimide Activated Kit, and two rabbits were immunized intradermally with Hunter's TiterMax Gold (CytRx, Norcross, GA) as adjuvant. After 5 weeks, rabbits were boosted intramuscularly, and after 10 days serum was obtained. The IgG fraction was purified from serum on a Protein A column (Pierce), and antibodies to ALDH6 were purified by affinity chromatography with the immunizing peptide immobilized on a Sulfolink column (Pierce).

Analysis of ALDH6 by Western blot was performed by separation of cytosol or recombinant protein samples (ALDH6 produced as described, or rat RALDH-2, a gift of Marcia Newcomer, Baton Rouge, LA) on 10% SDS-PAGE and electrophoretic transfer to polyvinylidene difluoride membrane. After blocking in 5% milk, blots were incubated with affinity-purified anti-serum at 1:200 dilution overnight at 4°C. Immunodetection was performed with horseradish peroxidase-conjugated secondary antibody against rabbit IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at 1:5000 dilution for 1 h, followed by detection in enhanced chemiluminescence reagents.

Blocks of normal human breast tissue obtained from reduction mammaplasty were generously provided by Rafael Mira-y-Lopez (Mount Sinai School of Medicine, New York, NY). Slides were prepared by sectioning, deparaffinized in xylene, and rehydrated. Antigen retrieval was performed by heating in 10 mM citrate (pH 6.0) for 12 min in a 1100 W microwave oven. Sections were washed in 20 mM Tris-HCl, 150 mM NaCl (pH 7.6), blocked in 3% BSA, and incubated with affinity-purified IgG against ALDH6 at 1:25 dilution or previously characterized affinity-IgG against CRABP(II) at 1:500 dilution (16) overnight at 4°C. After being washed, slides were incubated with biotin-conjugated secondary antibody, followed by anti-biotin IgG conjugated with alkaline phosphatase (both from Jackson ImmunoResearch, West Grove, PA). Brown staining was produced by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium/iodonitrotetrazolium violet substrate (Dako, Carpinteria, CA) for 20 min. Slides were then counterstained in hematoxylin and mounted with aqueous mountant from Serotec (Oxford, United Kingdom).

## RESULTS

**Synthesis of RA from Retinal Is Impaired in MCF-7 Cells.** We reported previously that the MCF-7 cell line was incapable of synthesis of RA when provided with retinol. The synthesis is a two-step process, with the first step being the oxidation of retinol to retinal, followed by oxidation of retinal to RA. MTSV1.7 cells, an immortalized but nontumorigenic breast epithelial cell line shown previously to be able to synthesize RA in culture when provided with retinol, were, as expected, also able to convert retinal to RA (Fig. 1). However, incubation of MCF-7 cells with retinal gave almost no detectable production of RA. MTSV1.7 cells produced an average of 370 ± 140

<sup>4</sup> Internet address: <http://prospector.ucsf.edu>.

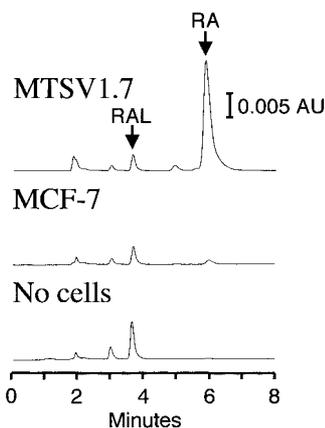


Fig. 1. Synthesis of RA from retinal by MTSV1.7 cells but not MCF-7 cells. MTSV1.7 and MCF-7 cells were incubated in culture medium containing  $2 \mu\text{M}$  retinal for 4 h. The cells and medium were then extracted, and RA content was analyzed by HPLC as described in "Materials and Methods." Shown are representative chromatograms demonstrating synthesis of RA by MTSV1.7 cells (*top*) but little, if any, synthesis by MCF-7 cells (*middle*). Retinal-containing medium incubated without cells showed little if any nonspecific oxidation of retinal to RA (*bottom*).

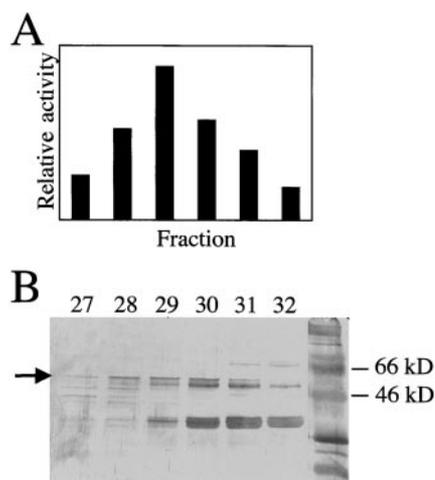


Fig. 2. Isolation of the RALDH activity in MTSV1.7 cells. The RALDH activity present in MTSV1.7 cytosol was partially purified by a two-column procedure as described in "Materials and Methods." Fractions from size-exclusion chromatography were assayed, identifying a single peak of activity eluting at a position consistent with a molecular weight of  $M_r$  100,000,000 (A). Analysis of these fractions by SDS-PAGE and Coomassie Blue staining revealed a  $M_r$   $\sim$ 56,000 band, the intensity of which most closely paralleled the level of RALDH activity (B, arrow).

pmol RA (average  $\pm$  SD;  $n = 7$ ) per confluent 60-mm dish of cells, compared with only  $10.2 \pm 0.5$  pmol RA ( $n = 3$ ) recovered from comparable cultures of MCF-7 cells. This indicates that at least part of the defect in the ability of MCF-7 cells to synthesize RA from retinol occurred at the second step of RA biosynthesis. Interestingly, mRNA for either of two known RALDHs, RALDH-1 and RALDH-2 (17–20), was not detected by RT-PCR in the MTSV1.7 cell line with primers able to detect specifically either rat or human mRNA. However, a band was observed with degenerate primers designed to detect any member of this family of ALDHs (data not shown). Sequencing of this product revealed the presence of mRNA for family member ALDH6 (14), which is proposed to be a RALDH but has not yet been confirmed at the time of this result. Nucleotide identity of the 565-bp RT-PCR product, excluding the primer sequences, and the ALDH6 sequence (GenBank accession no. HSU07919) was 100%.

**Identification of the RALDH in MTSV1.7 Cells as ALDH6.** To determine whether ALDH6 was indeed the enzyme responsible for the

cellular conversion of retinal to RA, cytosol was collected from MTSV1.7 cells and assayed, revealing potent RALDH activity. The activity was followed through a two-column purification procedure, migrating as a single component during the first separation on an anion-exchange HPLC column (data not shown). Fractions containing the activity were pooled and applied to a HPLC size-exclusion column. A single peak of activity was again observed, eluting at a position consistent with a molecular weight of  $M_r \sim$ 100,000,000 (Fig. 2A). The fractions containing activity (27–33) were then examined by SDS-PAGE. A number of bands were visible after protein staining, but only one, with an apparent molecular weight of  $M_r$  56,000, corresponded in intensity as judged by visual inspection with the measured activity in each fraction (Fig. 2B). Members of this ALDH

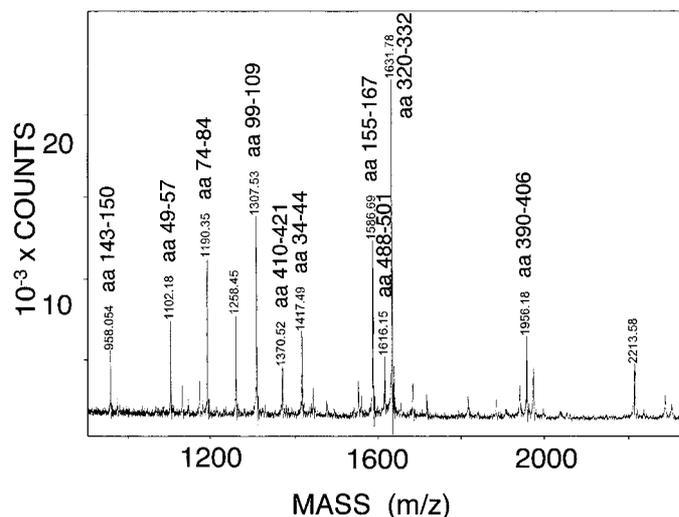


Fig. 3. Identification of the isolated RALDH as ALDH6 by mass spectrometry. The protein band identified as corresponding most closely to the pattern of RALDH activity was excised from the gel and digested with trypsin, and the mass of the tryptic peptides was determined by mass spectrometry analysis. Shown is the mass spectrogram of the digest, indicating the masses of the tryptic fragments. This pattern of masses was then matched to a theoretical tryptic digest of translated sequences in GenBank, using the ProteinProspector web-based application.<sup>4</sup> The closest matching tryptic fragment mass pattern was with ALDH6; shown above each mass peak is the predicted tryptic fragment of ALDH6. aa, amino acids.

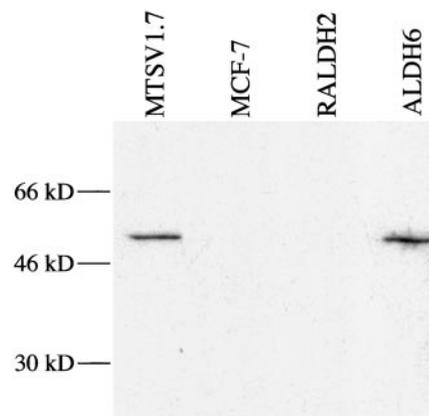


Fig. 4. Western blot analysis of MTSV1.7 and MCF-7 cytosol and specificity of the anti-ALDH6 antibodies. Polyclonal rabbit antibodies were raised against a peptide sequence of ALDH6 that was unique from known RALDHs 1 and 2. The affinity-purified IgG was used to probe a Western blot containing cytosol from MTSV1.7 cells (Lane 1) or MCF-7 cells (Lane 2) and recombinant rat RALDH-2 (Lane 3) and recombinant ALDH6 (Lane 4). The only immunoreactivity apparent was a band of the expected size,  $M_r \sim$ 56,000, recognized in MTSV1.7 cytosol but not found in MCF-7 cytosol or recombinant total ALDH6 protein. The antibodies did not cross-react with RALDH-2. kD, molecular weight in thousands.

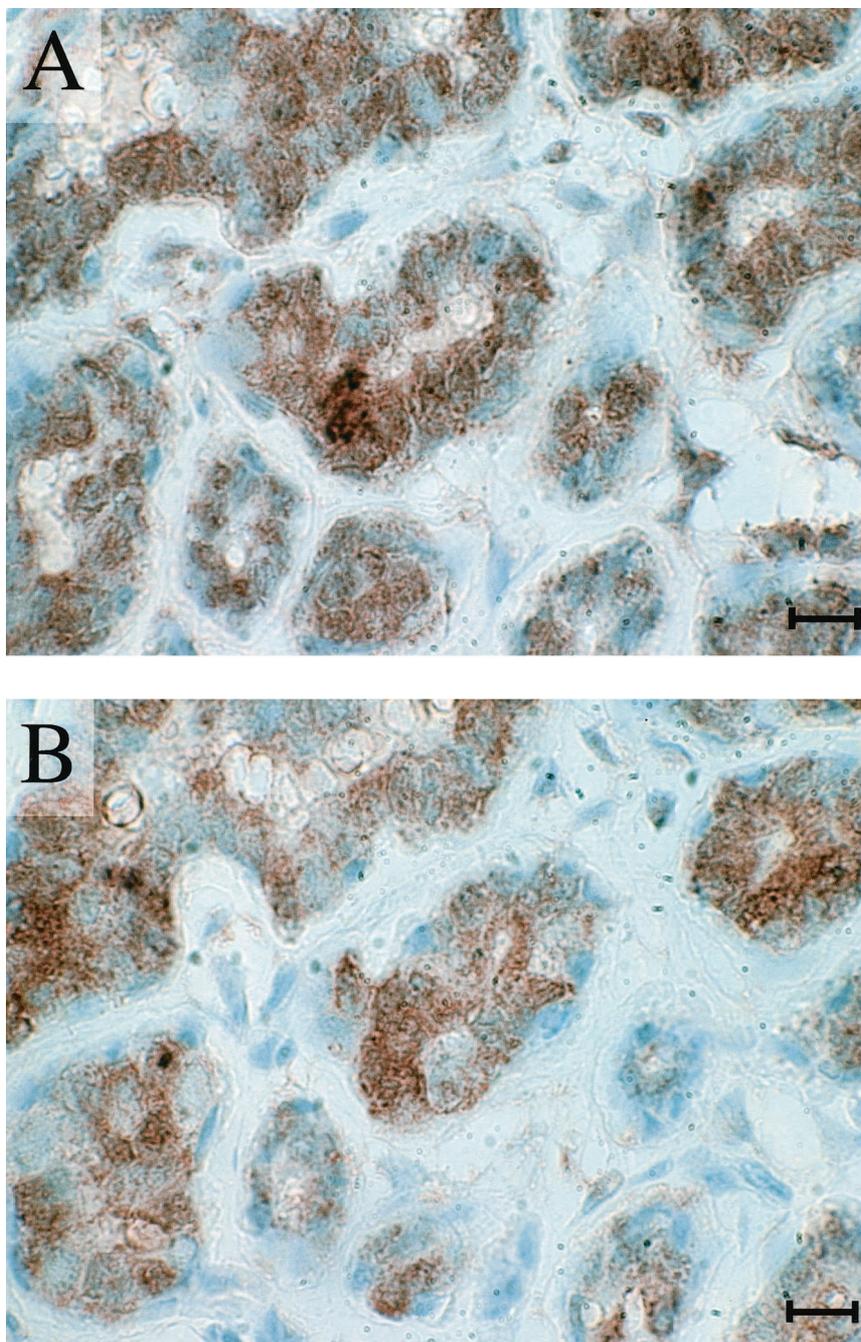


Fig. 5. Immunohistochemical staining of normal human mammary epithelia with antibodies to CRABP(II) and ALDH6. Tissue sections of normal human mammary gland were stained with the antibodies to ALDH6 used in Fig. 4 (A) or with antibodies raised previously against CRABP(II) (B). Antibodies were detected with alkaline phosphatase-conjugated anti-biotin and biotin-conjugated antirabbit antibodies; the *brown* color indicates positive staining. ALDH6 and CRABP(II) colocalized to the glandular epithelia of the breast. Staining with either antibody preparation is restricted to the cytoplasm of the epithelial cells. Bar, 10  $\mu$ m.

family are normally either dimers or tetramers. Under the conditions used here, the enzyme appeared to be a dimer, consistent with the shift in apparent molecular weight between the sizing column elution position and migration position after SDS-PAGE.

This band was excised and subjected to tryptic digestion, and the mass of the principal peptide products was determined by mass spectroscopy. This pattern of fragments was matched against the predicted masses of theoretical tryptic digests of known proteins. The band was identified as ALDH6 by this procedure because all but two of the observed peaks in the mass range examined corresponded (within 1 mass unit) to a predicted tryptic peptide from the ALDH6 sequence (Fig. 3). The murine homologue of ALDH6 (also termed RALDH-3) has now been cloned and is believed to be one of the RALDHs functioning in the embryo (21–23), consistent with our results here.

**Western Blot Analysis and Immunohistochemical Localization of ALDH6.** We produced polyclonal antibodies in rabbit against a peptide sequence from ALDH6 that was distinct from other known members of this family. Western blot analysis of cytosol from MTSV1.7 cells with the affinity-purified IgG population from the antiserum showed a single band of the appropriate size but no band for cytosol from MCF-7 cells (Fig. 4). The antibody preparation did not react with recombinant rat RALDH-2.

Expression of ALDH6 in normal human breast epithelium was demonstrated by immunohistochemical analysis with this antibody preparation (Fig. 5A). Staining was noted in the epithelial cells of the smaller glands and ducts, coinciding exactly with sites of CRABP(II) expression (Fig. 5B), the putative marker for this system of RA synthesis. No immunostaining was observed when the primary antibody preparation was omitted (data not shown).

**Transfection of ALDH6 into MCF-7 Cells Restores RA Synthesis.** Absence of expression of ALDH6 in MCF-7 cells suggested that their inability to catalyze the synthesis of RA from retinal was attributable to this difference from the MTSV1.7 cell line and from normal mammary epithelium. When human ALDH6 was transiently expressed in MCF-7 cells, they indeed gained the ability to oxidize retinal to RA (Fig. 6A). ALDH6-transfected cells produced an average of  $141 \pm 51$  pmol RA per 60-mm dish of cells ( $n = 4$ ), whereas vector-transfected cells produced only  $11.4 \pm 7.0$  pmol RA ( $n = 5$ ). This established the ability of ALDH6 to carry out this oxidation in a cellular environment in addition to its demonstrated activity in the test tube. Furthermore, ALDH6-transfected MCF-7 cells could make a small but significant amount of RA when provided with retinol, whereas normal MCF-7 cells or vector-transfected cells could not, suggesting the primary defect in RA biosynthesis lies in the second step (Fig. 6B).

As a further test of these results, we observed that, after transfection of ALDH6 into MCF-7 cells, cytosol from these cells gained the ability to convert retinal to RA in the *in vitro* assay (Fig. 7A) with an average activity of  $20.4 \pm 4.5$  pmol RA/mg protein/min ( $n = 8$ ). This compares well with an average activity of  $27.2 \pm 7.1$  pmol RA/mg protein/min from MTSV1.7 cytosol ( $n = 9$ ). This activity was absent

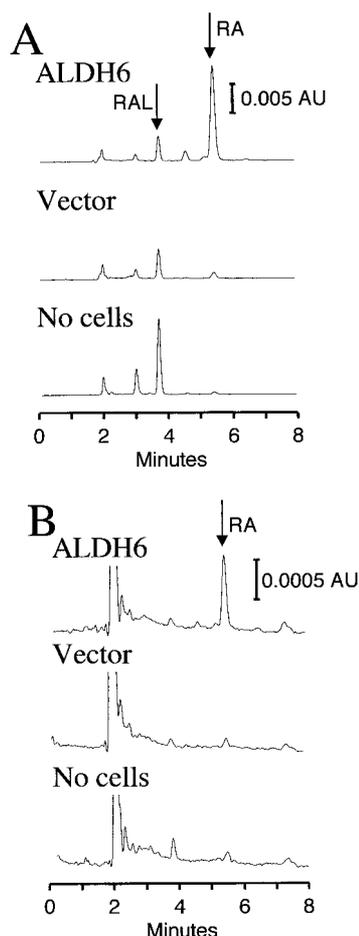


Fig. 6. Transient expression of ALDH6 in MCF-7 cells confers the ability to synthesize RA. MCF-7 cells were transiently transfected with the ALDH6 coding sequence in pcDNA3.1(-) or with vector alone. Thirty-six h after transfection, cells were provided with  $2 \mu\text{M}$  retinal or retinol for 4 h. After incubation with retinoids, cells and medium were extracted, and RA was analyzed by HPLC. Representative chromatograms show that MCF-7 cells transfected with ALDH6 have the ability to oxidize retinal to RA (A, top), whereas vector-transfected cells do not (middle). Furthermore, transfection of ALDH6 also conferred on the MCF-7 cells an ability to synthesize RA from retinol (B, top), whereas vector-transfected cells cannot (middle). Also shown is medium incubated with retinoids in the absence of cells (bottom).

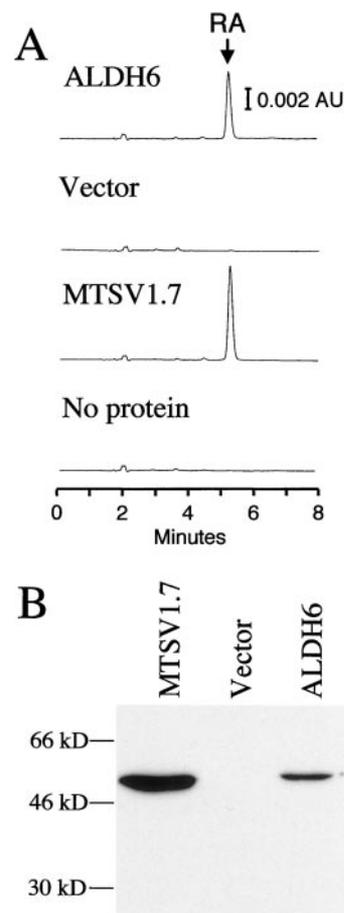


Fig. 7. ALDH6 activity *in vitro* in transiently transfected MCF-7 cells. MCF-7 cells were transfected as in Fig. 5, but cytosol was prepared from ALDH6-transfected cells, vector-transfected cells, and MTSV1.7 cells. Cytosolic protein ( $100 \mu\text{g}$ ) was incubated with  $2 \mu\text{M}$  retinal and  $100 \mu\text{M}$   $\text{NAD}^+$  for 20 min before reactions were stopped with ethanol, and RA was extracted and analyzed by HPLC. Only ALDH6-transfected MCF-7 and MTSV1.7 cell cytosols possessed RALDH activity, as seen by representative chromatograms showing the presence of RA (A). B, expression of ALDH6 in transfected cells was confirmed by Western blot analysis. Lane 1, MTSV1.7 cytosol; Lane 2, vector-transfected MCF-7 cytosol; Lane 3, ALDH6-transfected MCF-7 cytosol. The blot was probed with antibodies described in Fig. 4.

in normal or vector-transfected MCF-7 cells, because no detectable RA was produced by incubation of cytosol with retinal. No inhibition of activity was observed with the inclusion of cellular retinol-binding protein in the reaction, a protein that can bind retinal and is found in cells that synthesize RA (data not shown). Expression of ALDH6 in cytosol from transfected cells was further confirmed by Western blot analysis of the cytosolic fraction from transfected cells (Fig. 7B).

## DISCUSSION

Our previous work established that normal mammary epithelial cells have the ability to synthesize RA, an ability that was greatly reduced or lost in five of six cell lines derived from human breast cancers (13). An essential question is whether this loss had occurred prior to establishment of these lines, *i.e.*, does loss of the ability to synthesize RA potentially contribute to the development of some breast cancers? This cannot currently be addressed by measurement of RA itself, in biopsy material for example, because of technical difficulties. However, identification of the enzymes involved in the synthesis of RA from retinol will allow molecular biological and immunological techniques to be used to address the question.

Here we identified that the RALDH activity present in an immor-

talized but nontumorigenic human mammary cell line is the previously cloned human ALDH6 enzyme and that this enzyme is expressed in normal human breast epithelia. When transiently transfected into MCF-7 cells that are unable to convert retinal to RA and are deficient in expression of ALDH6, the cells gain the ability to synthesize RA from retinal as well as some ability to synthesize RA from retinol. These findings indicate that ALDH6 is the physiologically relevant enzyme involved in the biosynthesis of RA in mammary epithelium and that lack of expression of this enzyme in a breast cancer cell line was responsible for the inability to synthesize RA from retinol.

However, it should be stressed that synthesis of RA from retinol is a two-step process, with the conversion of retinol to retinal generally considered to be the rate-limiting step. The ability of the MCF-7 cells transfected with ALDH6 to convert retinal to RA was comparable with the ability of the MTSV1.7 cells to do this conversion. However, the production of RA from retinol was markedly less, indicating a potential problem in the first step. Identification of the enzyme responsible for the oxidation of retinol to retinal is necessary before one can consider any analysis of a correlation between loss of RA-synthesizing ability and cancer development. This is a current goal of this laboratory because we have detected none of the known rat retinol dehydrogenases in a rat mammary carcinoma cell line that is capable of synthesizing RA (NMU line),<sup>5</sup> indicating that the enzyme will be novel.

Several different lines of evidence (reviewed in Ref. 24) suggest that defects in the RA signaling pathway are involved in cancer, from the observation that a diet deficient in vitamin A leads to an increase in the number of tumors in animal models to the observation that RA itself can induce the differentiation and inhibit the growth of many tumor cells, as well as the identification that components of the RA signaling mechanism are absent in cancer cells. Particularly in breast cancer, a number of studies have shown aberrant expression of the nuclear receptors or of binding proteins for retinoids. Decreased expression of the  $\beta$  isoform of RAR was noted in several breast cell lines (25–28); the work by Jing *et al.* (28) also showed that expression of cellular retinol-binding protein was constitutively lower in breast cell lines compared with normal breast tissue. Xu *et al.* (29) noted that loss of RAR $\beta$  expression was progressive from normal tissue to premalignant lesions and lowest in invasive cancers. This may be of particular interest with regard to the work here in that RAR $\beta$  is directly induced by RA (30) and therefore raises the question of whether a defect in synthesis of RA is perhaps the more fundamental defect in some malignant transformations.

The sequence of these events, *i.e.*, loss of RA signaling, loss of receptor expression, and malignant transformation, remains to be elucidated, but to the growing body of evidence linking aberrant RA signaling and cancer we add the loss of ALDH6 expression in MCF-7 cells as the first molecular defect to be identified to explain loss of the RA-synthesizing phenotype in breast cancer cells.

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## REFERENCES

1. Wolbach, S. B., and Howe, P. R. Tissue changes following deprivation of fat-soluble A vitamin. *J. Exp. Med.*, **42**: 753–777, 1925.
2. Sporn, M. B., Clamon, G. H., Dunlop, N. M., Newton, D. L., Smith, J. M., and Saffiotti, U. Activity of vitamin A analogs in cell cultures of mouse epidermis and organ cultures of hamster trachea. *Nature (Lond.)*, **253**: 47–50, 1975.
3. Dowling, J. E., and Wald, G. The biological function of vitamin A acid. *Proc. Natl. Acad. Sci. USA*, **46**: 587–608, 1960.
4. Ong, D. E., Page, D. L., and Chytil, F. Retinoic acid binding protein: occurrence in human tumors. *Science (Wash. DC)*, **190**: 60–61, 1975.
5. Mark, M., Ghyselinck, N. B., Wendling, O., Dupe, V., Mascres, B., Kastner, P., and Chambon, P. A genetic dissection of the retinoid signalling pathway in the mouse. *Proc. Nutr. Soc.*, **58**: 609–613, 1999.
6. Napoli, J. L. 17 $\beta$ -Hydroxysteroid dehydrogenase type 9 and other short-chain dehydrogenases/reductases that catalyze retinoid, 17 $\beta$ - and 3 $\alpha$ -hydroxysteroid metabolism. *Mol. Cell. Endocrinol.*, **171**: 103–109, 2001.
7. Duester, G. Genetic dissection of retinoid dehydrogenases. *Chem. Biol. Interact.*, **130–132**: 469–480, 2001.
8. Chen, H., Howald, W. N., and Juchau, M. R. Biosynthesis of all-*trans*-retinoic acid from all-*trans*-retinol: catalysis of all-*trans*-retinol oxidation by human P-450 cytochromes. *Drug Metab. Dispos.*, **28**: 315–322, 2000.
9. Yoshida, A., Rzhetsky, A., Hsu, L. C., and Chang, C. Human aldehyde dehydrogenase gene family. *Eur. J. Biochem.*, **251**: 549–557, 1998.
10. Bucco, R. A., Zheng, W. L., Davis, J. T., Sierra-Rivera, E., Osteen, K. G., Chaudhary, A. K., and Ong, D. E. Cellular retinoic acid-binding protein(II) presence in rat uterine epithelial cells correlates with their synthesis of retinoic acid. *Biochemistry*, **36**: 4009–4014, 1997.
11. Zheng, W. L., Bucco, R. A., Sierra-Rivera, E., Osteen, K. G., Melner, M. H., and Ong, D. E. Synthesis of retinoic acid by rat ovarian cells that express cellular retinoic acid-binding protein-II. *Biol. Reprod.*, **60**: 110–114, 1999.
12. Zheng, W. L., Sierra-Rivera, E., Luan, J., Osteen, K. G., and Ong, D. E. Retinoic acid synthesis and expression of cellular retinol-binding protein and cellular retinoic acid-binding protein type II are concurrent with decidualization of rat uterine stromal cells. *Endocrinology*, **141**: 802–808, 2000.
13. Mira-y-Lopez, R., Zheng, W. L., Kupumbatti, Y. S., Rexer, B., Jing, Y., and Ong, D. E. Retinol conversion to retinoic acid is impaired in breast cancer cell lines relative to normal cells. *J. Cell. Physiol.*, **185**: 302–309, 2000.
14. Hsu, L. C., Chang, W. C., Hiraoka, L., and Hsieh, C. L. Molecular cloning, genomic organization, and chromosomal localization of an additional human aldehyde dehydrogenase gene, *ALDH6*. *Genomics*, **24**: 333–341, 1994.
15. Herr, F. M., and Ong, D. E. Differential interaction of lecithin-retinol acyltransferase with cellular retinol binding proteins. *Biochemistry*, **31**: 6748–6755, 1992.
16. Wardlaw, S. A., Bucco, R. A., Zheng, W. L., and Ong, D. E. Variable expression of cellular retinol- and cellular retinoic acid-binding proteins in the rat uterus and ovary during the estrous cycle. *Biol. Reprod.*, **56**: 125–132, 1997.
17. Bhat, P. V., Labrecque, J., Boutin, J. M., Lacroix, A., and Yoshida, A. Cloning of a cDNA encoding rat aldehyde dehydrogenase with high activity for retinal oxidation. *Gene (Amst.)*, **166**: 303–306, 1995.
18. Penzes, P., Wang, X., Sperkova, Z., and Napoli, J. L. Cloning of a rat cDNA encoding retinal dehydrogenase isozyme type I and its expression in *E. coli*. *Gene (Amst.)*, **191**: 167–172, 1997.
19. Wang, X., Penzes, P., and Napoli, J. L. Cloning of a cDNA encoding an aldehyde dehydrogenase and its expression in *Escherichia coli*. Recognition of retinal as substrate. *J. Biol. Chem.*, **271**: 16288–16293, 1996.
20. Zhao, D., McCaffery, P., Ivins, K. J., Neve, R. L., Hogan, P., Chin, W. W., and Drager, U. C. Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur. J. Biochem.*, **240**: 15–22, 1996.
21. Grun, F., Hirose, Y., Kawachi, S., Ogura, T., and Umesono, K. Aldehyde dehydrogenase 6, a cytosolic retinaldehyde dehydrogenase prominently expressed in sensory neuroepithelia during development. *J. Biol. Chem.*, **275**: 41210–41218, 2000.
22. Li, H., Wagner, E., McCaffery, P., Smith, D., Andreadis, A., and Drager, U. C. A retinoic acid synthesizing enzyme in ventral retina and telencephalon of the embryonic mouse. *Mech. Dev.*, **95**: 283–289, 2000.
23. Suzuki, R., Shintani, T., Sakuta, H., Kato, A., Ohkawara, T., Osumi, N., and Noda, M. Identification of RALDH-3, a novel retinaldehyde dehydrogenase, expressed in the ventral region of the retina. *Mech. Dev.*, **98**: 37–50, 2000.
24. Niles, R. M. Vitamin A and cancer. *Nutrition*, **16**: 573–576, 2000.
25. Roman, S. D., Clarke, C. L., Hall, R. E., Alexander, I. E., and Sutherland, R. L. Expression and regulation of retinoic acid receptors in human breast cancer cells. *Cancer Res.*, **52**: 2236–2242, 1992.
26. Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. Down-regulation of retinoic acid receptor  $\beta$  in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.*, **5**: 133–141, 1994.
27. van der Burg, B., van der Leede, B. M., Kwakkenbos-Isbrucker, L., Salverda, S., de Laat, S. W., and van der Saag, P. T. Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol. Cell. Endocrinol.*, **91**: 149–157, 1993.
28. Jing, Y., Zhang, J., Bleiweiss, I. J., Waxman, S., Zelent, A., and Mira-y-Lopez, R. Defective expression of cellular retinol binding protein type I and retinoic acid receptors  $\alpha 2$ ,  $\beta 2$ , and  $\gamma 2$  in human breast cancer cells. *FASEB J.*, **10**: 1064–1070, 1996.
29. Xu, X. C., Sneige, N., Liu, X., Nandagiri, R., Lee, J. J., Lukmanji, F., Hortobagyi, G., Lippman, S. M., Dhingra, K., and Lotan, R. Progressive decrease in nuclear retinoic acid receptor  $\beta$  messenger RNA level during breast carcinogenesis. *Cancer Res.*, **57**: 4992–4996, 1997.
30. de The, H., Marchio, A., Tiollais, P., and Dejean, A. Differential expression and ligand regulation of the retinoic acid receptor  $\alpha$  and  $\beta$  genes. *EMBO J.*, **8**: 429–433, 1989.

<sup>5</sup> Unpublished results.