Retinoids induce MMP-9 expression through RARα during mammary gland remodeling


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TO FEMALE MAMMALS, THE COST OF LACTATION is exorbitant compared with other physiological processes. Thus, to assure a good milk production, the lactating animal has developed several physiological changes that include hyperphagia, liver and mammary gland hypertrophy, increased cardiac output, and increased blood flow to the gland, which, together with widespread changes in the metabolism of different tissues, assure a sufficient supply of substrates to the gland for milk production.

Few adult tissues exhibit extensive apoptosis under physiological conditions. These include the small intestine, adipose, uterus, ovary, and mammary gland (1, 9). After being weaned, the mammary gland is remodeled in preparation for the next pregnancy/lactation cycle through a complex cellular program.

This weaning process has two phases. The first phase is reversible; it depends on p53 and is characterized by the disappearance of the physiological adaptations and by an increase in the number of apoptotic events in the epithelia of the lobulo-alveolar compartment (15, 33). The second phase is p53 independent; it is irreversible and includes a proteolytic degradation of the basement membrane and remodeling of the mammary gland. To achieve the latter events, two main families of extracellular matrix (ECM)-degrading proteinases are activated, matrix metalloproteinases (MMPs) and serine proteinases, that are involved in the activation of plasminogen to plasmin (11, 17). Recently, a third phase consisting of adipogenesis or biosynthesis has been ascribed to the regressing mammary gland (12).

MMPs are a broad family of more than 20 enzymes that are considered the key enzymes in the turnover of the ECM. Depending on their substrate specificity and domain similarity, MMPs are classified into collagenases, stromelysins, gelatinases, and membrane-bound MMPs (4, 22). The expression of MMPs is tightly regulated at the transcriptional and posttranscriptional levels by hormones, cytokines, and growth factors. Moreover, the activities of MMPs are also regulated by the presence of their inhibitors, tissue inhibitors of MMPs (TIMPs), and activators (plasmin, stromelysin, or MMP-2).

Thus, a balance between MMPs and TIMPs regulates epidermal cell function and morphology during mammary gland development. During lactation, MMP activities remain low, since an intact basement membrane is required for the secretory phenotype; however, MMPs are highly expressed after being weaned, when there is a remodeling process essential for postweaning involution of the mammary gland (14, 16).

Retinoic acid (RA) and its precursor retinol (vitamin A) are known to be involved in the maintenance, differentiation, and function of many epithelial tissues. The pleiotropic effects of these compounds are mediated by nuclear retinoid receptors, which have the potential to transduce the RA signal in vivo. This family of receptors includes the retinoid acid receptors (RARs), which bind both all-trans- and 9-cis-RA stereoisomers, and the retinoid X receptors (RXRs), which bind 9-cis-RA only. Each family is composed of three isotypes, designated α, β, and γ, encoded in separated genes. RARs and RXRs can form hetero- and homodimers that act as ligand-dependent transcription factors by binding to retinoid response elements in the promoters of RA target genes (7, 20, 21). In addition to the nuclear receptors, there are two cytoplasmic proteins in-
volved in retinoid signal transduction, cellular RA-binding protein (CRABP I and CRABP II) and cellular retinol-binding protein (CRBP). All of them play an important role in retinoid homeostasis; CRBP acts to chaperone retinol- and retinol-to-retinoid-metabolizing enzymes, and CRABP II is thought to regulate the access of RA to nuclear receptors (8), whereas CRABP I is instead believed to be involved in RA catabolism (26).

Retinoids have been shown to modulate production of MMPs and TIMPs in mammary epithelial cells (24), endothelial cells, and alveolar macrophages (6, 10). Numerous studies (23, 32) in cell lines regarding the role of retinoids in breast cancer have been published. In vivo studies have shown that RA is required for proper morphogenesis of the mammary gland (30); however, little is known of the physiological role of RA in mammary gland involution. Here, we report the over-expression of CRABP II and RARe during weaning, which suggests that RA is involved in the involution of this tissue. Additionally, to highlight the role of retinoids on ECM remodeling, treatment of lactating rats with retinol palmitate induced transient increases of plasma retinoid levels and generated a slight decrease in milk production together with an induction of MMP-9 mRNA via RARe.

MATERIALS AND METHODS

**Animals and tissue extraction.** Pregnant Wistar rats were kept in individual cages in a controlled environment (12:12-h light-dark cycle), and they were fed water and food ad libitum. The rats were cared for and handled in conformance with National Institutes of Health guidelines and the Guiding Principles for Research Involving Animals and Humans approved by the Council of the American Physiological Society. The Research Committee of the School of Medicine (University of Valencia, Valencia, Spain) approved the study protocol. Following parturition, litters were maintained with at least 8 pups. Then, on day 12 of lactation, the rats were divided into different groups: control lactating rats (n = 6) at the peak of lactation (days 12–15), weaned rats in which pups were removed 12 days after delivery to initiate involution (the weaning took place for 2, 4, 8, 24, and 72 h before death (≥3 rats for each condition were used)), and another group that included rats at the peak of lactation that had been weaned for 24 h plus resuckling for another 24 h (n = 3).

To determine the role of retinoids in mammary gland involution, four rats at the peak of lactation received a peritoneal injection of vitamin A (200,000 IU all-trans-retinol palmitate/kg body wt) 8 h before the experiment was performed. The trans-retinol palmitate (type VII, synthetic; Sigma-Aldrich, St. Louis, MO) was dissolved in 0.15 M NaCl. The control rats for the last group (n = 3) were injected with vehicle.

The rats were anesthetized with pentobarbital sodium (60 mg/kg body wt in 0.9% NaCl ip; Braun Medical, Rubi, Spain), and blood was collected from the aorta in heparinized syringes. Then, the inguinal mammary glands were removed and processed immediately.

**Milk production.** Milk production was measured as previously described (33). Briefly, the following equation relating pup milk yield to pup weight and weight gain was used to estimate milk production: yield = 0.0322 + 0.0667 (weight) + 0.877 (gain), where yield is daily yield per pup (g day⁻¹ pup⁻¹), weight is pup weight (g), and gain is daily pup weight gain (g/day).

**Retinoid determination.** Retinol and retinol palmitate were measured in serum according to the method described by Barua and Olson (3).

**RNA extraction and PCR analysis.** Total RNA from mammary tissue was extracted using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). RT-PCR was performed as described previously (34), using the SuperScript reverse transcriptase (Invitrogen) and the Biotools DNA polymerase gel form (B&M Laboratories, Madrid, Spain). CRABP II, CRBP-I, and MMP-2, -3, and -9 mRNA expression were studied by RT-PCR using specific oligonucleotide primers (Table 1). Products were separated by agarose gels (1.2% wt/vol) and stained with ethidium bromide.

**Quantitative real-time RT-PCR was performed in one step using the TTH DNA polymerase kit (Roche Diagnostics-Boehringer Mannheim, Mannheim, Germany) as instructed by the manufacturer. Real-time quantitations of mRNAs for CRABP II, CRBP-II, and MMP-2, -3, and -9 relative to cyclophilin A mRNA were performed using the iCycler iQ real-time PCR detection system (Bio-Rad, Munich, Germany) with SYBR Green I detection. Target cDNAs were amplified in separated tubes: 10 min at 95°C, and then 40 cycles of denaturation (95°C for 30 s), annealing, and extension (at 62°C for 1 min/cycle). The increase in fluorescence was measured during the extension step. The threshold cycle (CT) was determined, and then the relative gene expression was expressed as follows: fold change = 2^(ΔΔCT) , where ΔΔCT = CT target – CT housekeeping, and ΔΔCT = ΔCT treated – ΔCT control.

**Chromatin immunoprecipitation assays.** Chromatin from mammary tissue was fixed and immunoprecipitated according to Borrás et al. (8). Briefly, mammary tissue samples were excised and immersed in PBS, pH 7.4, and 1% formaldehyde for 8 min to crosslink the transcription factors to DNA. The reactions were stopped by adding glycine to a final concentration of 0.125 M. After centrifugation at 1500 g for 5 min, the cell pellet was resuspended in cell lysis buffer (85 mM KCl, 0.5% NP-40, 5 mM HEPES, pH 8.0) supplemented with a protease inhibitor cocktail (Sigma-Aldrich), incubated on ice for 15 min, and centrifuged at 3500 g for 5 min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.1) at a ratio 1:1 (vol/wt) relative to the initial tissue weight, incubated on ice for 10 min, aliquoted in 1-ml fractions, and stored at −80°C until use for chromatin immunoprecipitation (ChIP) assay.

**Cross-linked chromatin (1 ml of each sample) was sonicated on ice with 10 pulses of 10 s and 38% amplitude in a Vibra-Cell VCX-500 sonicator.** The average chromatin size of the fragments obtained was ~500 bp. The sonified chromatin was treated as described previously.

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**Table 1. List of oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRABP II</td>
<td>AGCTTCCATCATTTGGTCAG</td>
<td>ACTGGAAAGCATCCGATCG</td>
</tr>
<tr>
<td>CRBP-1</td>
<td>GGAAGATCTGAGACCAAGGAG</td>
<td>TTTGACGTTGCAACCTCTGTCG</td>
</tr>
<tr>
<td>MMP-2</td>
<td>GACCTTGACGACCAACATCG</td>
<td>GCTGTATTGCGAGCCCTGGAAAC</td>
</tr>
<tr>
<td>MMP-3</td>
<td>CTCATTGACGACCTCCACACAGAATC</td>
<td>GTGCCATGCGCTGGAAAAGCTTC</td>
</tr>
<tr>
<td>MMP-9 (promoter)</td>
<td>GTGAAACAGGTTGCTGAA</td>
<td>CAGGCTCTTTCGAGCGAGATT</td>
</tr>
<tr>
<td>MMP-9 (coding region)</td>
<td>GGATGTACACTCGCTGGCTT</td>
<td>TTGGTACGTTGAGATGCTGGG</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>AGACCTGGGAGGAGAAAGATT</td>
<td>CATGGCTCTTTCGACCTTGGC</td>
</tr>
</tbody>
</table>

CRABP, cellular retinoic acid-binding protein; CRBP, cellular retinol-binding protein; MMP, matrix metalloproteinase.
The antibodies used in this case were p300 and RARα (both from Santa Cruz Biotechnology, Santa Cruz, CA).

**PCR analysis of immunoprecipitated chromatin.** After DNA purification, input, immunoprecipitated, and unrelated antibody fractions were analyzed by PCR with the appropriate primer pairs to amplify products of 180–300 bp in length, corresponding to either the promoter or coding regions of the target genes (see Table 1). PCR fragments were size fractionated by 2% agarose gel electrophoresis and stained with ethidium bromide.

**Protein extraction and immunoblotting analysis.** The freeze-clamped tissue (0.1 g) was homogenized in 1 ml of extraction buffer as described previously (33). The lysates were normalized for protein concentration using a Bradford-based assay (Bio-Rad protein assay). Equal amounts of protein (10 μg) were loaded in SDS-PAGE gels and analyzed by Western blot, as described previously (33). Antibodies for CRABP II and CRBP-1 were a generous gift from Dr. Cecile Rochette-Egly and Dr. Christine Chaponnier, respectively. The rest of the antibodies were all from Santa Cruz Biotechnology. Equal loading was confirmed by tubulin expression and Coomassie staining.

**Gel zymography.** Gelatine zymography was carried out as described by Heussen and Dowdle (13). In brief, protein lysates (150 μg) were diluted in nonreducing SDS sample buffer (62.5 mM Tris·HCl, pH 6.8, 25% glycerol, 4% SDS, and 0.001% bromophenol blue) and separated on 10% SDS-PAGE minigels containing 1 mg/ml gelatin (Bio-Rad). After electrophoresis, the gels were washed for 30 min at room temperature in renaturing buffer (10 mM Tris·HCl, pH 7.5, 2.5% Triton X-100), incubated for 36 h at 37°C in enzyme buffer (50 mM Tris·HCl, pH 7.6, 200 mM NaCl, 5 mM CaCl2, 0.02% Brij-35), and stained with Coomassie Brilliant Blue R-250. Gelatino-lytic activities were detected after destaining by clear bands, indicating the lysis of the substrate.

**Statistics.** Data are presented as means ± SE. Statistical significance was evaluated with one-way ANOVA. The homogeneity of the variances was analyzed by the Levene test; in those cases in which the variances were unequal, the data were adequately transformed before ANOVA. The null hypothesis was accepted for all the values of these sets in which the F value was nonsignificant at P > 0.05. The data for which the F value was significant were examined by Tukey’s test at P < 0.05. In Fig. 7, statistical analysis was performed by the least significant difference test. After the analysis of the variances, the sets of data in which F was significant were examined by the modified t-test, using P < 0.05 as the critical limit.

**RESULTS**

Expression of retinoid-binding proteins during mammary gland involution. Retinoids exert their effect through retinoid-binding proteins. In the involuting mammary gland, both CRABP type II and CRBP-I mRNA levels appeared to be higher after 8-h weaning compared with the peak of lactation (Fig. 1A). To quantify this result, real-time RT-PCR was performed in control lactating rats and at different stages of weaning. The graphs show a sharp increase in CRABP II and CRBP-I mRNA after 72-h weaning. This increase is reversible, since mRNA expression diminished almost to control values during resuckling.

When analyzing the levels of both cellular retinoid carriers, CRBP-I and CRABP II, by Western blot, an increase in CRBP-I and CRABP II levels was shown after 24- and 72-h weaning (Fig. 1B). These values returned to control levels after resuckling. The time and rate of increase in protein levels were consistent with the increase in mRNA assessed by real time RT-PCR.

The effects of retinoids are mediated through the RAR signaling pathway. Retinoids mediate their biological activity by binding to specific nuclear receptors. To identify the receptors involved in the regulation of mammary regression, we performed immunoblot analysis in mammary tissue of lactating and weaned rats. Within the RAR subfamily, RARα is the most ubiquitously expressed, whereas RARβ and RARγ display more distinctive patterns of distribution (7). As shown in Fig. 2, RARα and RARγ protein levels were increased as early as 2-h weaning and remained elevated in involuting mammary glands. On the other hand, RARβ protein levels increased after 8-h weaning, reaching a peak after 24 h and decreasing there-
after. When studying the RXR subfamily, it was demonstrated that RXRs protein levels were also elevated during the weaning process (Fig. 2). Interestingly, this increase was reversed toward control values after resuckling.

**Induction of MMP-9 after weaning.** An increase in MMP-9 (92 kDa gelatinase, gelatinase B) in mammary epithelial cells upon RA treatment, mediated through an RARα-dependent signaling pathway, has already been reported (24). To determine whether weaning modulates MMP-9 expression through the RARα signaling pathway, real-time RT-PCR was performed in the mammary tissue from lactating and weaned rats. As seen in Fig. 3A, the MMP-9 mRNA expression showed a twofold increase after 24-h weaning, and this increase was even more evident in rats weaned for 72 h. In accordance with the previous result, the protein levels for MMP-9 were increased after weaning in a time-dependent manner, reaching a plateau at 72-h weaning (Fig. 3B).

**RARα induces MMP-9 mRNA expression after weaning.** To examine in vivo the potential role of RA on the expression of MMP-9, ChIP assays were performed with an affinity-purified antibody directed against RARα and the coactivator p300 (Fig. 4A). DNA was extracted from the input, bound, and unrelated antibody fractions; equal amounts from each fraction were amplified using primers specific for the MMP-9 promoter region. The binding was determined by the relative intensity of ethidium bromide fluorescence compared with the input control. Our data show that RARα binding to the MMP-9 gene was evident after 8-h weaning, reaching a maximum at 24 h, whereas it was almost undetectable during lactation. Regarding the RAR coactivator p300, it was bound to the MMP-9 gene at 8-, 24-, and 72-h weaning. The binding to the MMP-9 coding region, used as a negative control, was not observed (Fig. 4B), indicating a specific binding of these transcription factors exclusively to the MMP-9 promoter.

**Induction of other MMPs in involuting mammary gland.** MMPs are essential in the ECM remodeling that takes place during the involution of the mammary gland. Indeed, MMP-9 is a key proteinase governing the degradation of basement membrane collagen as well as different types of gelatine. In this context, we have also studied the temporal secretion profile of other ECM-degrading MMPs, MMP-2 (72 kDa gelatinase or gelatinase A) and MMP-3 (stromelysin-1), during weaning. Real-time RT-PCR analysis was performed in rats at the peak of lactation and in weaned rats (Fig. 5A). The results revealed that the amounts of MMP-2 and MMP-3 mRNAs were significantly higher after 24-h weaning than those of lactating rats. This induction is time dependent and seems to diminish after the resuckling of the litter.

To determine whether the higher expression of MMPs was correlated with protein levels, we performed Western blot studies (Fig. 5B). Evaluation of protein expression revealed that MMP-3 was significantly increased after 24-h weaning and remained high for longer periods. The same result was observed for MMP-2, although in this case a second band of 55 kDa, corresponding to the active form of the enzyme, appeared as early as 8-h weaning and was at maximum at 72 h. In contrast with the result obtained by RT-PCR, this protein increase was not reversed after resuckling for either MMP-2 or MMP-3.
Temporal activity of gelatinases A and B in mammary gland involution. MMPs are synthetized as latent zymogens, which have to be biologically activated by cleavage of their proenzyme peptides (29). Both pro-MMP and its active form are bound by TIMP-1, retarding activation and proteolytic activity of these MMPs. To determine whether the higher expression of MMPs was correlated with increased enzyme activity, we performed zymography studies. As seen in Fig. 6, there was a significant increase in gelatinase activities (MMP-2 and/or MMP-9) after weaning. Activation of pro-MMP-2 (70-kDa band) to an active form occurred at 72-h weaning. The upper band (105 kDa) likely represents pro-MMP-9 and also appears after 72-h weaning. This increased enzyme activity correlates with mRNA and protein levels in the weaned mammary tissue (Figs. 3 and 5).

Protein analysis by Western blot revealed that the inhibitor TIMP-1 decreased during weaning and was almost undetectable after 72-h weaning (Fig. 6B), which explains the increase in MMP-2, -3, and -9 levels.

MMP-9 induction is dependent on retinoid increase. To elucidate the physiological role of RA on MMP-9 induction, control lactating rats were treated with an acute dose of retinol palmitate over a period of 8 h. In these rats, the concentration of this compound, measured by HPLC, was significantly higher in arterial blood compared with controls (data not shown). The binding of RAR to MMP-9 promoter was studied using the

Fig. 4. In vivo association of p300 and RARα with the MMP-9 promoter. Results of a representative chromatin immunoprecipitation (ChIP) experiment, performed in samples from C lactating mammary gland and after different times of weaning (W 8 h, W 24 h, and W 72 h), are shown. Chromatin was immunoprecipitated with anti-p300, anti-RARα antibodies, or a nonrelated antibody anti-IgG as control. An aliquot of the input chromatin is also shown. A: primers specific to the promoter region for MMP-9 gene were used to amplify the DNA isolated from the ChIP assay. B: the PCR products obtained with oligonucleotides specific for the MMP-9 coding region were examined as negative controls.

Fig. 5. Increased levels of MMP-2 and -3 during mammary gland involution. A: expression of MMP-2 and -3 mRNA in the mammary gland from C lactating rats, weaned rats (8, 24, and 72 h, respectively), and W+R rats was determined by real-time RT-PCR. Cyclophilin A was used as internal control (see MATERIALS AND METHODS). Results are means ± SE for 3 independent experiments. ANOVA was performed for the statistical analysis, where different superscripted letters indicate significant differences, P < 0.05; always the lowest value within the group. B: Western blot analysis of whole protein extracts (10 μg) derived from mammary tissue of C lactating rats, weaned rats (W 2 h, W 4 h, W 8 h, W 24 h, and W 72 h), and 24-h W+R rats were probed with specific antibodies against MMP-2 and MMP-3. Immunoblots are representative of 3 separate experiments. Equal loading was confirmed by rehybridizing the blot with antibody against α-tubulin and by Coomasie staining.
immunoprecipitation of complex RAR-DNA with purified antibodies reactive to RARα and the coactivator p300. DNA was extracted from the input, bound, and unrelated antibody fractions; equal amounts from each fraction were analyzed using primers that amplify the MMP-9 promoter region. Our data show that the RAR and p300 binding to the MMP-9 gene was almost undetectable in control lactating rats injected with vehicle, whereas in acute retinol palmitate treatment the binding was significantly higher (Fig. 7A). The binding to the MMP-9 coding region, used as a negative control, was not observed (Fig. 7B), indicating a specific binding of these transcription factors exclusively to the MMP-9 promoter. Finally, real-time RT-PCR demonstrated that this RARα binding to MMP-9 led to a higher expression of this gene in rats treated with retinol palmitate (Fig. 7C).

DISCUSSION

Retinoic acid, the natural metabolic derivative of vitamin A, has been shown to be an important molecule involved in the regulation of cell proliferation, differentiation, and apoptosis. Its physiological role in different tissues has been widely studied (20, 21). In mammary gland these studies have been of particular interest, since a defective biosynthesis of RA from retinol has been demonstrated in breast cancer cell lines compared with nontransformed mammary epithelial cells (23, 27). Moreover, defective RA signaling has been associated with breast cancer development (8, 32). Indeed, RA is known to have an essential role in the regulation of the different stages of mammary gland development, such as puberty, pregnancy, and lactation (4, 24, 30). Nonetheless, little is known about the physiological role of this molecule during mammary gland involution.

The mammary gland is a highly dynamic organ that undergoes growth and remodeling during pubertal development and lactation. Both systemic and local hormones regulate the development and morphogenesis of the mammary gland. At the...
end of lactation, in response to diminishing lactational stimuli and milk stasis, the mammary gland undergoes involution that involves extensive epithelial apoptosis and tissue remodeling (11, 12). This process consists of a primarily reversible phase of apoptosis, a secondary irreversible phase of apoptosis and tissue remodeling, and a tertiary phase of adipogenesis. These stages bring about the disappearance of milk-secreting acini and the reversion of the gland to a virgin-like state. The first stage is considered proteinase independent and p53 dependent (15, 33). It entails milk accumulation, induction of genes involved in mediating apoptosis, and initial apoptosis of epithelial cells. During this early phase, there was a strong induction of p53 and other genes participating in cell cycle arrest and apoptosis (33). However, very low levels of MMPs were present in the gland, whereas TIMP-1 was upregulated, shifting the proteolytic balance in favor of inhibition. The second proteinase-dependent stage begins around day 3 of involution and is characterized by a decrease in lactogenic hormones, collapse of glandular structures, and basement membrane destruction, resulting in active tissue remodeling and irreversible loss of the differentiated functions of the mammary gland (9, 11, 12, 18). This phase is characterized by upregulation of mRNA and activity for proteolytic enzymes, including gelatinases A and B and stromelysin-1, and down-regulation of the inhibitor TIMP-1 (18). The third stage, recently termed the biosynthetic stage, involves adipose differentiation and reconstitution. This process can be artificially achieved by forced removal of the pups.

In this context, we have found that the RA signaling pathway is fully activated during involution, assessed by the increased expression of retinoid-binding proteins and nuclear RA receptors. As previously mentioned, it has been demonstrated (8) that retinoids exert their effects through retinoid-binding proteins, facilitating the delivery of RA to RAR and thereby enhancing the transcriptional activity of the receptors. These observations suggest that upregulation of direct RAR target genes will be increased by CRABP II. As shown in Fig. 1, our results demonstrated that the retinoid carrier proteins CRABP II and CRBP-I were sharply increased during weaning compared with lactating glands. Moreover, the mRNA levels for CRABP II were increased 10-fold after 72-h weaning (Fig. 1A), in good agreement with previously published Affymetrix data (34). Interestingly, we found that both mRNA and protein levels reverted to control values after resuckling, suggesting a reversible mechanism of action of retinoids at this phase of involution.

In parallel with these results, retinoid nuclear receptor (RARα, RARγ, and RXRα) protein levels were increased as early as during 2-h weaning, remained elevated in involuting mammary glands, and yet again reverted towards control values after resuckling (Fig. 2). On the other hand, RARβ increase is delayed, reaching a sharp peak at 24 h and declining thereafter. Retinoid receptors have already been studied at different stages of mammary gland development; in this sense, it has been described that RARγ is strongly expressed throughout mammary gland development (30), whereas RARβ is expressed only at specific time points (30). It has been suggested that RARγ may be responsible for RA signaling in nulliparous and pregnant mammary gland, whereas a combination of RARβ and RARγ may be required for RA signaling in lactating mammary gland. Our results suggest that, most likely, this could also state for mammary gland involution. Interestingly, although RARα protein levels are barely detected in virgin, pregnant, or lactating mammary gland (30), our results demonstrate that this protein is strongly induced during mammary gland involution. Taken together, these data suggest that different RA receptors function in distinct phases of the mammary gland development, assigning RARα a prominent role during mammary gland regression, since this is the only stage where RARα is expressed in mammary gland.

Regarding the biological significance of these findings, the prominent role played by RARs in the modulation of MMP expression has been demonstrated extensively (24, 30). Matrix-degrading proteinases clearly have an important function as mediators of the tissue remodeling that occurs during mammary gland involution. Previous work has detailed the expression of secreted MMPs in mammary gland development; MMP-2, -3, and -9 are present in the mammary tissue and play a role not only in branching morphogenesis (16, 31) but in involution as well (2, 18). Indeed, some of the mouse strains deficient for specific secreted MMPs display altered mammary tissue morphology or involution (31). However, few studies (28) have focused on the regulation of MMP expression during involution, being difficult to predict which factors induce the expression of MMPs. The data presented here show a relationship between RA and MMP-9 expression, suggesting an important role of retinoids in the development and involution of glandular epithelial structures. Using chromatin immunoprecipitation assay, we demonstrated for the first time that RARα, although absent from MMP-9 promoter in lactating controls, is recruited to the MMP-9 promoter in mammary gland during weaning (Fig. 4). Moreover, it has been shown that p300 acts as a necessary cofactor mediating nuclear receptor-activated gene transcription. Herein, we demonstrate p300 binding to the MMP-9 promoter. These data correlate with the transcription of the gene as seen by quantitative RT-PCR, in which the expression was increased severalfold after 24- and 72-h weaning (Fig. 3). Overall, these results strongly suggest that RARα and p300, as integrators of the signaling pathway, modulate the expression of MMP-9 during mammary gland involution.

ECM remodeling is a well-orchestrated process in which it is likely that a functional overlap exists between different MMPs. In our experimental conditions we have also seen an upregulation of other proteinases, such as MMP-3 and MMP-2, during the mammary gland remodeling (Fig. 5). However, in the case of these two MMPs, the mRNA and protein levels did not return to control values after the resuckling of the litter, as occurs with MMP-9. Previous publications (25, 30) have described MMP-3 to be downregulated by RAR, although the specific RAR involved in this modulation is not known. It has been suggested (25) that one of the members of the RAR family is competing with activating protein-1 for the DNA binding site. Possibly, as mentioned above, RARs function in a differential manner in distinct phases of the mammary gland development. Additional studies would be necessary to elucidate the precise mechanisms governing the different pattern of expression of MMP-3 by RAR throughout mammary gland development. On the other hand, the increase in MMP-2 expression, although not a target gene for RA, emphasizes the importance of a tight coordination between matrix-degrading enzymes, since it presents the same pattern of expression as MMP-3.
The regulation of proteases in the mammary gland can be achieved in three different ways: at the mRNA transcription level (1), at the zymogen activation level (2), and at the specific inhibitor level (15). Herein, we demonstrate that the higher level (1), at the zymogen activation level (2), and at the specific achieved in three different ways: at the mRNA transcription

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