Cleavage of Rat Prolactin Occurs Within Rat Mammary Tissue, and the Cleaved Form is Present in Serum but Not in Milk

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Abstract. **The present study was undertaken to determine whether cleavage of rat prolactin (rPRL) occurs within rat mammary tissue and to investigate whether the cleaved form and its 16-kDa amino terminal fragment are present in rat serum and milk. The relative abundance of the different forms of rPRL was determined by SDS-PAGE and immunoblot analysis using an antiserum to the 16-kDa fragment. Explants of mammary glands from lactating rats were incubated with intact rPRL. The tissue was then washed extensively to remove external PRL forms. The ratios of cleaved to intact rPRL in mammary tissue homogenate, in mammary-conditioned medium, and in serum from lactating rats were 4.2, 0.44, and 0.16, respectively. Although intact rPRL was found in milk, the cleaved form was not detectable. The 16-kDa fragment of rPRL was not detected in concentrated extracts of either serum or milk. These results indicate that lactating rat mammary tissue can internalize and cleave intact rPRL, but the cleaved form does not appear in milk. The cleaved rPRL is present in rat serum, but no evidence was obtained for the presence of the 16-kDa fragment of rPRL in either serum or milk.** [P.S.E.B.M. 1994, Vol 206]

Previous studies have shown that acid proteases in target tissues of rat prolactin (rPRL) can cleave the intact hormone to form a 2-chain derivative which, upon reduction, yields fragments that were assigned molecular masses of about 16 kDa and 8 kDa, based on SDS-PAGE analysis (1–10). Cleaved PRL is also present in and produced by rodent pituitary tissue (11–15), and it has been detected in human pituitary tissue and plasma (16–18). In addition, it was reported that the cleaved form and the 16-kDa fragment of PRL are present in rodent serum (19). Both cleaved rPRL and the 16-kDa fragment retain some of the biological activities of the intact hormone, and they are reported to have novel activities (15, 20). Thus, cleavage and reduction of rPRL may be of physiological significance. Cleavage of rPRL by target tissues may function to provide modified forms of the hormone for subsequent delivery elsewhere by serum or milk. In addition, cleaved rPRL may have functions in the cells that produce it.

When rPRL is incubated with explants of rat tissue *in vitro*, the cleavage that occurs is tissue-specific. Only mammary tissue yielded 16-kDa and 7-kDa fragments after reduction (5). Furthermore, the rate of cleavage of intact rPRL varied with the physiological state of the mammary gland whether the hormone was incubated with explants (5) or with a lysosomal fraction (4). Recently, we identified the sites of cleavage of rPRL by the mammary enzyme, and presented evidence that the cleaving enzyme is probably cathepsin D (21). Furthermore, mass spectrometric analysis

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showed that the two fragments had masses of ~16 kDa and ~6 kDa atomic mass units (21).

Several rat tissues secrete PRL-cleaving activity *in vitro,* and it is present in rat serum (5). Thus, it may be secreted by a variety of tissues *in vivo*. However, the cleaving enzyme in medium and serum is inactive at physiological pH. Acidification below pH 5 is required to activate it. Thus, the cleavage that occurs when rPRL is incubated with rat mammary tissue *in vitro* presumably occurs within intracellular compartments that can be acidified. The experiments reported in this paper were undertaken to determine whether rPRL is cleaved and retained within rat mammary explants *in vitro*. We also attempted to confirm the report that the cleaved hormone and its 16-kDa fragment are present in rat serum (19), and we investigated whether these forms are present in rat milk.

Materials and Methods

Animals. The Long-Evans rats used in these experiments were obtained from Simonsen Laboratories (Gilroy, CA). They were maintained in an environmentally controlled room (23° ± 1°C; 12:12-hr light: dark cycle), with food and water provided *ad libitum*. Their care, use, and maintenance were in strict accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals. All experimental procedures were described in a protocol that was approved by our Institutional Animal Care and Use Committee.

Mammary Tissue Incubation. Slices of mammary tissue from 14-day lactating rats were prepared as described previously (5). After rinsing to remove damaged cells and other debris, the slices were incubated in Hank's/20 m*M* HEPES medium (100 mg tissue/ml) under 95% O_2–5% CO_2 at 37°C in a gyratory shaker in 25-ml Erlenmeyer flasks containing 5 ml of the medium with rPRL at 5 μg/ml. After 2 hr, the tissue slices were removed and rinsed four times with swirling for ~3 min per rinse in 50 ml of PRL-free medium to remove residual extracellular rPRL forms. The tissue was then defatted and dehydrated by several washes of acetone at room temperature. To determine the protein content/mg dried, defatted tissue, a portion of the dried tissue was homogenized in 0.025 *M* Tris-HCl at pH 7.4 (4 mg/ml) in a polytron homogenizer, then assayed by the method of Hartree (22). For electrophoresis, the tissue was homogenized in Laemmli's sample buffer (23) containing β-mercaptoethanol at a concentration of 0.5 mg protein equivalent/μl sample buffer and 25 μg of protein were loaded on each gel lane for processing by SDS-PAGE (23). The PRL-containing medium in which the tissue slices were incubated was also processed by SDS-PAGE, as described (5). The protein bands were then transferred and immunostained (5).

Serum and Milk Extraction. Blood and milk were collected from lactating rats between Day 13 and 17 postpartum and the milk infranatant was prepared by a modification of the method of Grosvenor and Whitworth (24). In brief, 10 ml of whole milk were centrifuged at 15,000*g* for 5 min at 22°C, then the centrifuge tube was placed on ice for 10 min to solidify the upper lipid layer. Five milliliters of the infranatant between the lipid layer and the particulate pellet were transferred by pipette to an ultracentrifugation tube, which was spun at 100,000*g* for 30 min at 4°C. The second infranatant was transferred to other tubes and frozen.

The PRL in serum or milk infranatant was extracted and concentrated by a modification of a procedure that was originally developed to extract hormones from sheep pituitary glands (25). Five volumes of a solution of 97.6% acetone–2.4% concentrated HCl were added to each sample of serum or milk infranatant. This mixture was stirred at room temperature for 1 hr, then centrifuged at 2,000*g* for 5 min at room temperature, and the precipitate was discarded. The supernatant was combined with 37.5-vol (relative to the initial sample volume) of ice-cold absolute acetone, thoroughly mixed, and allowed to incubate at 4°C for 30 min. The mixture was centrifuged at 2,000*g* for 5 min at 0°C, the bulk of the supernatant was decanted, and the residual supernatant was allowed to evaporate. The dried precipitate was then solubilized with 0.55 ml of 0.1 *M* $NaHCO_3$ and desalted on PD-10 columns (Pharmacia-LKB, Piscataway, NJ) using a 1 m*M* NH_4HCO_3 buffer. The desalted mixture was lyophilized and resuspended in SDS-PAGE sample buffer prior to electrophoresis and Western blotting. The amount of extract applied to each column was derived from 1.0 ml of the original serum or milk infranatant.

This extraction method was evaluated to determine the percent recovery of added PRL and the maximum volume of test sample that could be analyzed per gel lane. Rat PRL was added to a pool of serum from hypophysectomized male rats, to a concentration of 1 μg/ml, then the hormone was extracted, as described above. This serum pool had only a trace of endogenous, extractable rPRL, which was several orders of magnitude lower in amount than the quantity of rPRL added. In a separate trial, we determined whether the extraction procedure would allow recovery of the 16-kDa rPRL fragment if it were present in rat serum using the following protocol: intact rPRL was cleaved by cathepsin D and the cleaved molecules were reduced, as described (4). Intact 23-kDa rPRL and the cleaved-reduced fragments were added to 0.2 ml of the

pool of serum from the hypophysectomized male rats to give concentrations of 1.0 μg/ml for the intact hormone and approximately 0.5 μg/ml of the 16-kDa fragment. The serum samples were extracted by the method described above. A sample of serum from the same pool with no added PRL forms was also extracted. Extract equivalent to 50 μl of the original serum was added to a gel lane and subjected to reducing SDS-PAGE prior to immunoblotting with the antiserum to the 16-kDa fragment.

Densitometric Analysis. The relative amounts of intact and cleaved rPRL in the samples were determined by densitometric analysis of the stained bands using a Soft Laser Scanning Densitometer SL-504-XL (Biomed Instruments, Inc., Fullerton, CA). The amount of the cleaved form was determined by measuring the quantity of the 16-kDa fragment present after SDS-PAGE under reducing conditions. The serum extracts were electrophoresed under reducing and nonreducing conditions and the tissue homogenates and milk infranatant extracts were processed under reducing conditions only. Our previous work showed that the 16-kDa fragment of rPRL was not present in incubation medium (5) or in tissue (unpublished) under nonreducing SDS-PAGE.

Results

Homogenates of mammary explants from lactating rats that were incubated without added PRL contained no detectable forms of the hormone. By contrast, cleaved and intact forms were found in the homogenate of the thoroughly rinsed mammary explants that were coincubated with added rPRL (Fig. 1). The ratio of cleaved to intact rPRL in the tissue ranged from 2.7 to 6.7, with a mean of 4.2 ± 1.0 (n = 3). The ratio of cleaved to intact rPRL in the incubation medium was only 0.44 ± 0.01 (n = 4). The tissue homogenate presumably included mainly internalized forms since most or all external rPRL forms should have been washed away by the extensive rinsing of explants before homogenizing.

Extracts of milk and serum from lactating rats (n = 4 for each) contained significant amounts of intact rPRL when run on nonreducing SDS-PAGE, but none of them contained detectable amounts of the 16-kDa fragment (results not shown). When electrophoresed under reducing conditions, only the serum extract contained detectable amounts of the 16-kDa fragment, which was presumably derived from the cleaved form (Fig. 2). Densitometric analysis of western blots revealed that the ratio of cleaved to intact rPRL in serum ranged from 0.09 to 0.19, with a mean value of 0.16 ± 0.03 (n = 4). Quantitative analysis of the extraction procedure showed that 95% of the added intact rPRL was extracted from the serum, and the hormone remained intact. The degree of concentration achieved was approximately 800- to 1000-fold. The immunoblot procedure used could detect intact rPRL in quantities of 0.5 ng/lane or less.

Reducing SDS-PAGE immunoblot analysis of the extract of the control serum and of the samples to which the intact and the 16-kDa and 6-kDa fragments of rPRL were added showed that the serum without added PRL had no detectable 23-kDa rPRL, but the samples to which the 23-kDa or the 16-kDa forms were added yielded a substantial amount (Fig. 3). Densitometric analysis showed that recovery of the added 23-kDa rPRL was almost 100%, while about 70% of the 16-kDa fragment was recovered.

Discussion

Our previous study showed that when rPRL was incubated with slices of rat mammary tissue, the cleaved form of the hormone appeared in the medium even though the incubation conditions were maintained at a pH of ~7.4 (5). When rPRL was incubated in medium in which rat mammary tissue had been previously incubated, the tissue-free medium did not

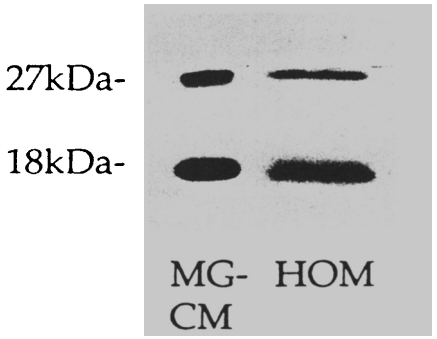

Figure 1. Western blot showing the rPRL content of mammary gland explants from lactating rats that were incubated with rPRL (5 μg/ml for 2 hr, thoroughly rinsed, and homogenized (HOM) prior to electrophoresis under reducing conditions. For comparison, acidified mammary gland-conditioned medium (MGCM) in which rPRL was incubated was run in an adjacent lane.

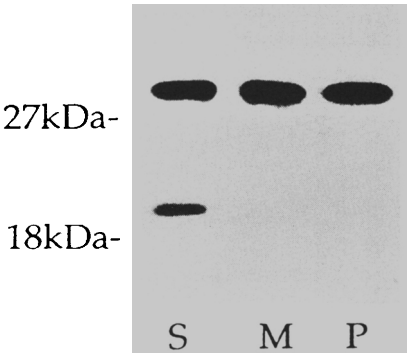

Figure 2. Western blot showing the PRL content of extracted serum (S) and milk infranatant (M) from a lactating rat compared with the purified rPRL standard (P). SDS-PAGE was run under reducing conditions.

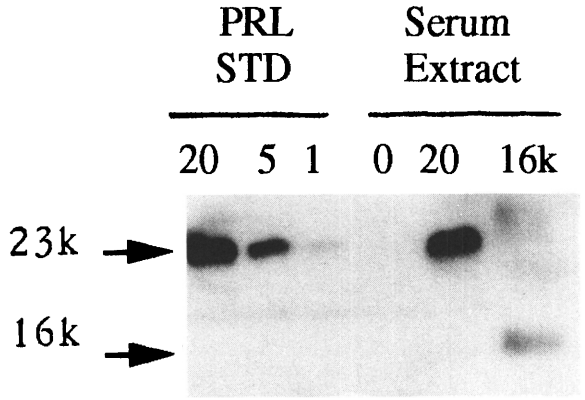

Figure 3. Western blot showing rPRL standards at 20, 5, and 1 ng/lane and rPRL in extracts of male rat serum. The "0" was from serum with no added rPRL and the "20" represents extracts estimated to contain 20 ng of the hormone. The 16-kDa lane contains extract from serum to which the 16-kDa fragment was added at approximately 0.5 μg/ml. Extract derived from 50 μl serum was run on each lane.

cleave the hormone when its pH was maintained at ~7.4, but cleavage did occur when the medium was acidified (5). Thus, although the cleaving enzyme was secreted by the tissue into the medium, the cleavage that occurred when the rPRL was incubated with the tissue at physiological pH could not have occurred in the medium. Accordingly, it may have occurred on the surface of cells or within them.

The results presented in this paper indicate that rPRL is taken up into rat mammary explants *in vitro* and that cleavage of the hormone probably occurs within the tissue. However, some of the intact and cleaved hormone that was detected in the extensively washed tissue may have been bound to extracellular surfaces. Although we have not identified the presumptive intracellular compartment where cleavage occurs, it seems likely that it would take place in endosomes or lysosomes. These vesicles contain cathepsin D (26), which cleaves rPRL at the same two sites as the mammary enzyme (21), and they can become acidic (27, 28). Some of the cleaved hormone is released into the extracellular spaces in the mammary tissue as it appears in significant amounts in the medium. However, although appreciable amounts of intact rPRL are transferred to milk (29), none of the cleaved form was detectable in the milk infranatant. Accordingly, the release of the cleaved form from mammary tissue may be unidirectional towards the vascular compartment.

Although cleaved PRL was not detectable in rat milk, cleavage of the hormone may occur in the stomach of suckling pups. Cleaving activity is present in rat milk (4), and the stomach of the pups would provide the acidic environment necessary for activating the enzyme. Indeed, our preliminary results indicate that milk in the stomach of pups does contain substantial amounts of the cleaved hormone (unpublished). Whitworth and Grosvenor (29) reported that PRL in rat milk is absorbed into the blood of suckling pups, and they presented evidence that milk PRL plays an important role in the regulation of the development of the hypothalamic tubero-infundibular dopaminergic neuronal system (30). However, the methods that they used to demonstrate absorption of rPRL across the gut of the pups (29) did not allow determination of the proportion of intact to cleaved hormone that appeared in the blood of the pups.

Our finding with the extract of the serum samples confirms the reports that cleaved PRL is present in serum or plasma of humans (16–18) and rodents (19). However, unlike Torner *et al.* (19), we failed to detect the 16-kDa fragment in serum, and none was detected in milk. The reason for the discrepancy between our present results and those of Torner *et al.* (19) is not evident at this time. The procedure that we used to concentrate the intact and cleaved forms of rPRL in serum was also very effective at recovering the 16-kDa fragment (Fig. 3). Thus, if it is present in the circulation its concentration must be very low (i.e., less than 0.5 ng/ml). Some evidence indicates that the 16-kDa fragment of rPRL has angiolytic activity (20). Accordingly, if 16K is formed *in vivo* to regulate angiogenesis or other functions, its production and actions may be entirely local.

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