# Comparison of the Ability of Normal Mouse Mammary Tissues and Mammary Adenocarcinoma to Cleave Rat Prolactin

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*Abstract.* **Previous studies have shown that target tissues cleave rat prolactin (rPRL) to form a two-chain derivative that yields ~ 16- and ~ 6-kDa fragments upon reduction. Both cleaved rPRL and the purified 16-kDa fragment have novel biological activities. Thus, cleavage may be of physiological significance. We determined whether normal mammary tissue or stroma (lacking mammary epithelial cells [MEC]) or mammary tumor tissue could cleave rPRL *in vitro*. Tumor explants were derived from a transplantable mammary tumor cell line (TPDTMT-4EP). The hormone was incubated with explants of those tissues from female BALB/c or DDD mice. Medium samples were processed by SDS-PAGE, and the relative abundance of intact and cleaved rPRL was determined by densitometric analysis of immunoblots using an antiserum to the 16-kDa fragment of rPRL.**

**After 2-hr of incubation, normal DDD mammary explants cleaved ~ 25% of added rPRL, but tumor explants cleaved none. Explants of intact virgin BALB/c mouse mammary gland and of parenchyma-free "cleared" mammary fat pad cleaved rPRL equally well, but explants of abdominal adipose tissue had low cleaving activity. Homogenates of cleared mammary fat pads that were incubated with rPRL contained a high proportion of the cleaved form but none of the free 16-kDa fragment. These results indicate that cleavage of rPRL is highly specific to mammary stroma. Such cleavage may yield a form of the hormone that has novel activities within the mammary gland or elsewhere in the body.** [P.S.E.B.M. 1995, Vol 208]

Several studies have shown that target organs of prolactin (PRL) in the rat can cleave the hormone to form a two-chain derivative (1–8). The cleaving enzyme is probably cathepsin D or a similar protein and the sites of cleavage have been identified (9). After reduction of the disulfide bond that connects the two chains, fragments of ~6 and ~16 kDa are obtained (10). The smaller one was originally thought to have a mass of ~8 kDa based on SDS-PAGE analysis (10). Cleaved forms of PRL are present in pituitary extracts or pituitary-conditioned medium of several species (10–13) and in the serum of rats, mice, and humans (11, 13–15). Thus, cleavage of the hormone apparently occurs *in vivo*. Although the cleaved form is present in the serum of lactating females in significant amounts, it was not detectable in rat milk (15).

Some evidence indicates that cleavage of rPRL may be of physiological significance. After cleavage by mammary lysosomes or explants, the hormone is relatively stable as it undergoes little additional processing (4, 5). By contrast, related hormones of the PRL-GH-PL family are either degraded into multiple fragments or are not cleaved by the mammary enzymes (4). Furthermore, cleavage of rPRL by rat mammary tissues varies with the physiological state of the gland (4, 5), and the cleavage apparently occurs within the tissue rather than on cell or tissue surfaces (15). A cleaved form of rPRL that is secreted by pituitary cells *in vitro* is reported to have a novel activity:

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It stimulates DNA synthesis in gonadotropes and thyrotropes (16). In addition, the 16-kDa fragment of rPRL has also been shown to have a unique activity: It inhibits endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (17, 18).

The physiological significance of PRL cleavage by target tissues is not known. Cleaved rPRL retains mitogenic activity on Nb2 and mammary epithelial cells (6, 19). Thus, it may play a role in normal mammary growth or in mammary tumorigenesis. In addition, as cathepsin D is apparently the enzyme responsible for the cleavage (9), and since that enzyme may play a role in the invasiveness of mammary cancers (20), the possibility exists that the cleaved form of PRL may be associated with some of the invasive actions of that enzyme.

In the present study, we compared the abilities of normal mammary tissue and stroma, and mammary tumor tissue to cleave rPRL *in vitro*. The ability of explants of abdominal adipose tissue to effect cleavage was also determined. Our results indicate that cleaving activity is a relatively specific function of the mammary stroma.

## Materials and Methods

**Explant Preparation.** The rudimentary duct system was removed from the mammary fat pads of 3-week-old BALB/c female mice by the method of DeOme et al. (21). Thus, the "cleared" fat pads consist only of stroma when the animals are adults. Intact or "cleared" mammary fat pads were removed when the mice were 4 months old and explants were prepared and cultured for 8 days in groups of 10 by procedures described in detail previously (22). The explants were cultured for 4 days in medium containing ovine (o) PRL (1 μg/ml) to allow them to adapt to the *in vitro* conditions prior to the addition of rPRL in place of the oPRL. Thus, cells damaged by cutting had time to recover or die. The medium was changed every 2 days, and the oPRL was replaced by rPRL (NIH) on the 4th or 6th day of culture at concentrations of 1–5 μg/ml. The medium was removed on the 6th and 8th day. The cross-reactivity of the oPRL with the antiserum used for western blots was less than 0.1% of that for rPRL, and Clapp (4) showed that the mammary enzymes process oPRL to yield multiple fragments, rather than the 6- and 16-kDa derivatives. Thus, the oPRL would not confound the detection of rPRL forms by immunoblotting. Explants of intestinal adipose tissue were prepared from the same animals and incubated similarly.

The mammary adenocarcinomas, which consisted mostly of neoplastic alveolar tissue, were obtained from subcutaneously transplanted TPDMT-4EP cells in DDD mice, according to the method of Matsuzawa and Yamamoto (23). When the tumors were 0.5–1.0 cm in diameter, they were removed along with the host's mammary gland, and the PRL-cleaving ability of explants of the two tissues was compared. Explants were rinsed four times with 25–50 ml fresh incubation medium to remove residual, extracellular PRL. They were then incubated in a swirling culture for up to 4 hr at 37°C using Hanks' salts with 20 m*M* HEPES at pH 7.4, with added rPRL at 5 μg/ml, as described previously (5). Since the amount of fat per unit wet tissue mass varied among tumor and mammary gland tissues, the amount of tissue used for incubations was normalized for the different tissue types on the basis of tissue protein mass per volume of medium, as described (5).

Explants were rinsed four times with 25–50 ml fresh incubation medium to remove residual, extracellular PRL. They were then defatted and dehydrated by several washes of acetone. Protein determinations were made on the basis of micrograms protein per milligram defatted and dehydrated tissue following homogenization in 25 m*M* Tris-HCl pH 7.5, with a polytron homogenizer. Samples of the mammary gland homogenate containing 25 μg protein were loaded on each gel lane for separation by SDS-PAGE. Protein bands were then transferred and immunostained as described previously (5).

Conditioned medium was prepared by incubating tissue in medium without added PRL for 2 hr followed by centrifugation at 15,000*g* for 10 min and collection of the supernatant. For acidification of this medium, the buffer was exchanged for one that could be more easily acidified (150 m*M* NaCl, 1 m*M* $NH_4HCO_3$, pH 7.8) using prepacked PD-10 columns (Pharmacia LKB, Uppsala, Sweden). The medium was then adjusted to pH 3.5 using citric acid-sodium phosphate buffer. Prolactin was added (5 μg/ml) and the medium was then incubated at 37°C for 2 hr.

**Western Blots.** Aliquots of incubation medium or tissue homogenate were analyzed by 15% SDS-PAGE. Prior to electrophoresis, medium samples in which tissue and PRL were co-incubated were centrifuged at 15,000*g*, after which the supernatants were desalted using PD-10 columns (1 m*M* $NH_4HCO_3$, pH 7.8 buffer), lyophilized, and solubilized in reducing sample buffer. Following incubation of conditioned medium samples, the medium was neutralized with 0.1 *N* NaOH. Medium samples containing added PRL in the range of 50–100 ng or samples of mammary gland homogenate containing 25 μg total protein were loaded on each gel lane. Protein bands were then transferred and immuno-stained, and the amount present in each lane was measured by densitometry, as described previously (5). The data are presented as the mean ± SEM. Tissue samples were obtained from at least three different animals in each case.

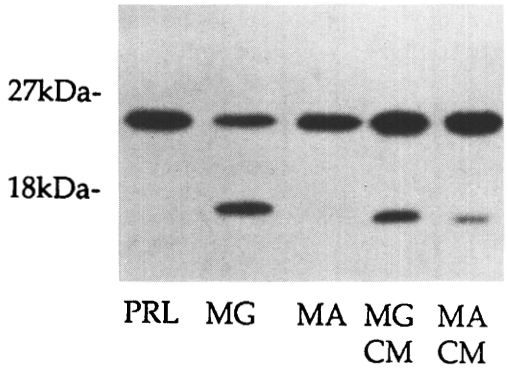


**Figure 1.** Western blot comparing the extent of rPRL processing during a 2-hr incubation with explants of normal DDD mouse mammary gland (MG) and mammary adenocarcinoma tissue (MA), and acidified mammary gland-and mammary adenocarcinoma-conditioned medium (MGCM and MACM, respectively). Rat PRL standard is designated PRL. The SDS-PAGE was run under reducing conditions.

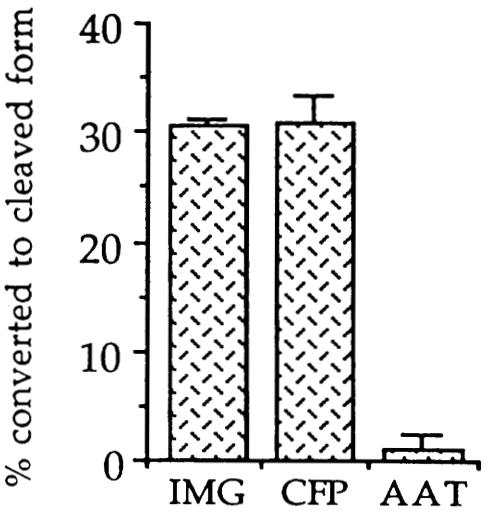


**Figure 2.** Densitometry data (mean ± SEM; $n = 3$ in each case) comparing the relative *in vitro* rPRL-cleaving ability of intact mammary gland (IMG), cleared mammary fat pad (CFP), and abdominal adipose tissue (AAT) from BALB/c mice.

The care, use, and maintenance of the mice used in these experiments 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.

## Results

Densitometric analysis of the western blots showed that after 2 hr of incubation, explants of normal DDD mouse mammary gland cleaved 26.7% ± 2.2% ($n = 3$) of the rPRL in the medium. By contrast, the tumor explant medium contained no cleaved rPRL (Fig. 1). Following acidification, the mammary-conditioned medium cleaved 19.4% ± 1.8% ($n = 3$) of

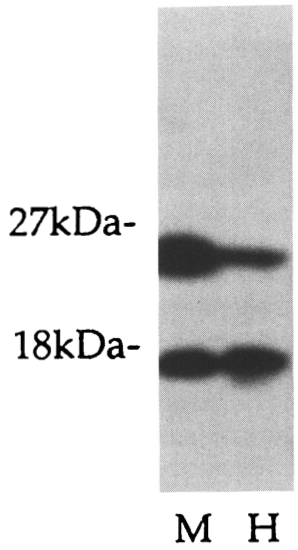


**Figure 3.** Western blot showing the rPRL content of cleared mammary fat pads from virgin BALB/c mice that were incubated with rPRL for 2 days, thoroughly rinsed, and homogenized prior to electrophoresis (H). For comparison, acidified, mammary gland-conditioned medium (M) in which rPRL was incubated was run in an adjacent lane.

the added rPRL, whereas the tumor-conditioned medium resulted in only 2.4% ± 1.0% ($n = 3$) conversion (Fig. 1). Without acidification, conditioned medium had no cleaving activity (data not shown). Clearly, although only mammary gland explants showed cleaving activity at physiological pH, both tissue types release the cleaving enzyme into the medium. However, the normal tissue released much more of the activity than did the tumor tissue.

Cleared and intact mammary explants showed equally high cleaving activity, but abdominal adipose tissue had very little activity (Fig. 2). Densitometric analysis of Western blots of medium conditioned by either abdominal adipose tissue or cleared mammary fat pad explants, in which rPRL was incubated after lowering the pH to 3.5, showed that the former cleaved >2% of the added rPRL, whereas the latter cleaved 40%–55% during a 1-hr incubation. Reducing electrophoresis of homogenates of cleared fat pad explants that were incubated with added rPRL and washed extensively contained a considerable amount of both cleaved and intact forms (Fig. 3); the ratio of cleaved to intact PRL ranged from 1.6 to 4.3, with a mean of 2.7 ± 0.7 ($n = 3$). Analysis under nonreducing conditions showed the absence of the 16-kDA fragment. Thus, the rPRL was cleaved but not reduced by the explants.

## Discussion

For the studies reported herein, we chose mouse mammary tissues because of the large variety of mouse strains that are available with differing mam-

mary gland characteristics and tumorigenic potentials. Rat and mouse PRL are highly homologous (24) and cleaved PRL has been identified in mouse pituitary glands and serum (11). Thus, mouse mammary tissues would presumably cleave rPRL in the same way as the homologous tissues or hormone.

The results obtained with the mouse mammary adenocarcinoma explants in organ culture indicate that tumor tissue has a limited capacity to cleave rPRL *in vitro*. This finding stands in sharp contrast to results obtained with the explants of intact rat (5, 15) and mouse (Fig. 1) mammary glands, which apparently internalize and cleave significant amounts of PRL before releasing it into the medium.

The experiments that compared the cleaving activity of explants of intact and cleared mammary fat pads showed why there is a difference between the normal mammary and tumor explants. The gland-free explants had as much cleaving activity as did the intact ones (Fig. 2). Thus, the MEC are not necessary for cleavage to occur. In fact, preliminary (unpublished) results obtained with normal mammary epithelial and mammary tumor cells cultured in collagen gel showed that both cell types contained small amounts of rPRL, that a high percentage of the internalized hormone was cleaved, and that some of it was reduced to yield the 16-kDa fragment. However, neither of these two mammary cell types released detectable amounts of processed rPRL into the incubation medium. Taken together, these results indicate that the mammary stroma is responsible for the large amount of cleaved rPRL that appears in medium after incubation of the hormone with explants of intact mammary tissue. Accordingly, the limited capacity of the tumor explants to cleave rPRL is probably due to the paucity of stromal cells in that tissue.

The finding that explants of abdominal adipose tissue did not cleave rPRL, whereas those of the mammary fat pad were highly active, indicates that this activity may be specific for mammary stromal cells. Accordingly, cleavage of PRL by the mammary stroma may serve to regulate the action of the hormone on MEC or in the fat pad. Intact PRL is mitogenic and lactogenic in MEC in cultures that lack stromal cells (22). Thus, the stroma is not necessary for PRL to have these effects. Furthermore, cleaved rPRL was about equipotent to the intact hormone as a MEC mitogen (19). Hence, cleavage did not increase the hormone's potency in that regard. Nevertheless, our results indicate that the MEC can cleave the intact hormone and reduce it to form the 16-kDa fragment. It remains to be determined whether this cleavage and/or reduction are necessary for PRL to exert effects on MEC.

Cleaved rPRL was not detectable in rat milk, but it is present in high concentrations in the serum of lactating females (15). Thus, the possibility exists that cleavage of the hormone by the mammary stroma serves to produce a novel regulator that functions elsewhere in the body.

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