

# Cathepsin D released by lactating rat mammary epithelial cells is involved in prolactin cleavage under physiological conditions

Mustapha Lkhider<sup>1</sup>, Roberta Castino<sup>2</sup>, Edwige Bouguyon<sup>3</sup>, Ciro Isidoro<sup>2</sup> and Michèle Ollivier-Bousquet<sup>3,\*</sup>

<sup>1</sup>Faculté des Sciences, Université Chouaib Doukkali, B.P. 20 El Jadida, Morocco

<sup>2</sup>Dipartimento di Scienze Mediche, Università 'Amedeo Avogadro', via Solaroli 17-28100, Novara, Italy

<sup>3</sup>INRA, Unité Génomique et Physiologie de la Lactation, 78352 Jouy-en-Josas CEDEX, France

\*Author for correspondence (e-mail: ollivier@jouy.inra.fr)

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## Summary

The 16 kDa prolactin fragment arises from partial proteolysis of the native 23 kDa prolactin pituitary hormone. The mammary gland has been involved in this processing, although it has not been clarified whether it occurs in stroma or epithelial cells or extracellularly. Also, the processing enzyme has not been defined yet. Here we show that the incubation medium of stroma-deprived mammary acini from lactating rat contains an enzymatic activity able to cleave, in a temperature- and time-dependent fashion, the 23 kDa prolactin to generate a 16 kDa prolactin detectable under reducing conditions. This cleavage was not impaired in the presence of hirudin, a thrombin inhibitor, but strongly weakened in the presence of pepstatin A, a cathepsin D inhibitor. Cathepsin D immuno-depletion abolished the capability of acini-conditioned medium to cleave the 23 kDa prolactin.

Brefeldin A treatment of acini, a condition that largely abolished the apical secretion of milk proteins, did not impair the secretion of the enzymatically active single chain of cathepsin D. These results show that mature cathepsin D from endosomes or lysosomes is released, likely at the baso-lateral site of mammary epithelial cells, and that a cathepsin D-dependent activity is required to effect, under physiological conditions, the cleavage of 23 kDa prolactin in the extracellular medium. This is the first report demonstrating that cathepsin D can perform a limited proteolysis of a substrate at physiological pH outside the cell.

Key words: Proteolysis, Hormone, Mammary gland, Lactation, Endosomes, Lysosomes

## Introduction

Prolactin (PRL), an hormone with a wide variety of biological activities in the regulation of reproduction, osmoregulation and immunomodulation (Ben Jonathan et al., 1996; Bole-Feysot et al., 1998; Freeman et al., 2000; Goffin et al., 2002), exerts important effects on the function of the mammary gland including effects on the expression of a number of genes and on the secretion of lipids and milk proteins (Hennighausen et al., 1997; Ollivier-Bousquet, 1998).

PRL exists in the pituitary and in serum in several molecular forms, some of them arising from alternative splicing of the PRL mRNA, other forms arising from post-translational processing of the full-length 23 kDa PRL, such as glycosylation, phosphorylation, polymerisation and proteolytic cleavage (Sinha, 1995). At least some of these post-translational modifications may also occur in the target tissues of the hormone (Clapp, 1987; Baldocchi et al., 1992). In lactating mammary tissue and in milk, in various animal species, the 23 kDa form, the 25 kDa glycosylated form and a number of smaller molecular forms of PRL have been detected (Sinha, 1995; Ellis and Picciano, 1995; Lkhider et al., 1996). Whether and how this molecular heterogeneity reflects the functional diversity of PRL effects remain largely obscure (Corbacho et al., 2002). In particular, a 16 kDa fragment of

PRL has received considerable attention (Corbacho et al., 2002). The N-terminal 16 kDa fragment of PRL acts as a potent inhibitor of angiogenesis in vivo and in vitro (Ferrara et al., 1991; Clapp et al., 1993; Struman et al., 1999) and as an apoptotic factor (Martini et al., 2000). It also induces the expression of plasminogen activator inhibitor 1 and it inhibits the urokinase activity (Lee et al., 1998). A C-terminal 16 kDa fragment of human PRL that is not angiostatic has also been generated in vitro (Khurana et al., 1999). These data underline the interest in defining the tissue and the metabolic pathways responsible for the generation of the 16 kDa PRL.

Based on in vitro assays, at least two proteases, namely thrombin and lysosomal cathepsin D (CD), have been considered good candidates for PRL processing enzymes in vivo. Thrombin was initially thought as the physiological processing enzyme, based on the facts that it acts at neutral pH and it is localised on endothelial cell surface, i.e. a site compatible with the physiological working place of the 16 kDa PRL. CD, the other candidate for in vivo processing of PRL, has been shown, in vitro at acidic pH, to convert intact rPRL into a 16 kDa N-terminal fragment and a 7 kDa C-terminal fragment linked by a disulfide bridge. After reduction the free N-terminal fragment is released. The two sites of cleavage occur at Tyr145 and Trp148 (Wong et al., 1986; Baldocchi et

al., 1993). In vitro at acidic pH, rat PRL could be cleaved by extracts from rat mammary tissue (Baldocchi et al., 1992) and by microsomal pellets from MCF-7 cells (Khurana et al., 1999), and it was suggested that the 16 kDa PRL was generated by CD within the lysosomes.

Huge quantities of pituitary PRL is released in the circulation at each milking during lactation. From blood, PRL is carried through the mammary epithelial cells (MECs) by transcytosis and is released into milk as intact and cleaved forms (Sinha, 1995; Ollivier-Bousquet, 1998). During this intracellular transport very little PRL is sorted to lysosomes (Seddiki and Ollivier-Bousquet, 1991; Seddiki et al., 2002). Consequently, the cleavage of PRL in these organelles might be relatively limited. Moreover, while the 14 kDa form of PRL can be detected in rat mammary tissue, the presence of the 16 kDa PRL in this tissue has not been demonstrated (Lkhider et al., 1996; Lkhider et al., 1997). It can be postulated that the cleavage occurs somewhere else during the transport of 23 kDa PRL from the capillaries to its receptor on the epithelial targets. Yet, the presence of mature CD in the extracellular environment of mammary gland has not been described. Moreover, this protease is believed to require a very acidic pH to exert its activity, a condition that is unlikely to be found in the pericellular space.

For a better comprehension of the physiological significance of PRL cleavage by the mammary gland during lactation, it is important to characterise the molecular forms that are generated, the protease implied, and to determine the intra- or extracellular site where the cleaving process takes place. In this study, enzymatically dissociated acini, cleared from endothelial and stroma cells and enriched in MECs, were prepared. We provide evidence that a cleavage of 23 kDa PRL occurs in the extracellular medium at physiological pH and that it requires the presence of mature CD released by the MECs. The secretion of CD and the CD-dependent cleavage also occur when MECs are exposed to brefeldin A (BFA), a condition that inhibits the apical secretion of milk proteins. The findings that, in vitro at pH 7.4, purified CD can generate the 16 kDa fragments of PRL and that in mammary gland acini CD largely accumulate at the basal region give support to the possible role of this lysosomal protease in the physiological processing of PRL.

## Materials and Methods

### Animals

Mammary gland of Wistar rats at day 13-14 of lactation, weighing 180-250 g, originating from our laboratory were used. The animals were housed at constant temperature (20°C) under a fixed cycle of 12 hours light and 12 hours dark, with free access to food and water. The ethical aspect of animal care complied with the relevant guidelines and licensing requirements laid down by the Ministère de l'Agriculture, France. All the experiments described below have been repeated on at least three rats.

### Materials

Hanks' medium was obtained from Gibco (BRL-Life Technologies, Cergy-Pontoise, France). Rat PRL (rPRL) and anti-rPRL antiserum were a kind gift from A. F. Parlow (National Hormone and Pituitary Program, Baltimore, MD). High specificity of this anti-rPRL antiserum in our experimental conditions has been shown previously (Lkhider et al., 1996). The antibody against rat TGN38 was kindly

provided by G. Banting (Bristol, UK). The anti-rat CD polyclonal antibody was produced in C. Isidoro's laboratory (Isidoro et al., 1995a). The antibody against mouse milk protein RAM/MSP was purchased from Nordic Immunological Laboratories, Tilburg, The Netherlands). This antibody is raised against total milk proteins and consequently recognises caseins plus proteins of the lactosera. Nitrocellulose membranes were purchased from Schleicher and Schuell (Dassel, Germany). The enhanced chemiluminescence detection kit was from Amersham (UK). Cathepsin D from bovine spleen and all other reagents were obtained from Sigma.

### Preparation and incubation of mammary gland fragments and acini

Mammary tissues from lactating rats dissected free of connective and adipose tissues were cut into small fragments (1-2 mm<sup>3</sup>). Incubations were performed in Hanks' medium plus sodium bicarbonate 0.2 g/l under an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The pH of the Hanks' medium after oxygenation was 7.5 and remained the same in the presence of mammary tissues during the whole incubation time. For the preparation of enzymatically dissociated acini, mammary fragments were incubated for 90 minutes at 37°C in Hanks' medium containing 200 UI/ml collagenase IV and 200 UI/ml hyaluronidase, washed and filtered through a strainer. Isolated cells were separated from the acini by three successive decantations for 15 minutes at 20°C in Hanks' medium. Mammary fragments or acini were preincubated in Hanks' medium in the presence or absence of 10 mM ammonium chloride, 10 µM chloroquine (fragments) or 5 µM BFA (acini) for 30 minutes at 37°C as indicated. Some fragments were further incubated in the presence or absence of 5 µg/ml rPRL for 15 to 30 minutes at 20°C in order to accumulate the hormone intracellularly, always in the presence of weak bases and then further incubated for 60 minutes at 37°C. Some fragments were extensively washed with Hanks' medium then chased in the same medium at 37°C. Other fragments were incubated in the presence of the hormone for 60 minutes at 37°C.

### Preparation of conditioned media and incubation with rPRL

Mammary fragments or acini were incubated in Hanks' medium for 60 minutes at 37°C under an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The conditioned media were obtained after either centrifugation at 15,000 g for 10 minutes or filtration through 5 µm filters. The same results were obtained with the two procedures. To study the processing of the 23 kDa PRL, 1 µg/ml of rPRL was incubated in the following conditions. (1) 60 minutes at 37°C in fresh medium. (2) 0 minutes to 24 hours at 37°C in conditioned medium. (3) 60 minutes at 37°C either in citrate phosphate buffer pH 3.2 in the presence of bovine CD (enzyme to protein ratio 1:200) or in 0.1 M Tris buffer pH 7.4 in the presence of bovine CD (enzyme to protein ratio 1:200) or in the presence of 12 U/ml thrombin. In parallel samples rPRL was incubated in the presence of these proteases plus 25 µM pepstatin A and 15 U/ml hirudin, respectively. In addition, PRL was also incubated in the presence of CD plus hirudin at pH 7.4. (4) 60 minutes at 4°C in conditioned medium. (5) 60 minutes at 37°C in conditioned medium previously boiled at 100°C for 10 minutes. (6) 60 minutes at 37°C in conditioned medium in the presence of 10 µM pepstatin A or 15 U/ml hirudin. (7) 60 minutes at 37°C in conditioned medium immunodepleted of CD. The latter was prepared by incubating 200 µl of conditioned medium with 2 µl of anti-rat CD antiserum for 16 hours at 4°C. Protein-A sepharose was then added for 60 minutes at 20°C with continuous rotating agitation at room temperature, then the mixture was spun at 10,000 g for 5 minutes. The supernatant corresponded to CD-depleted conditioned medium. To verify the nature of immunoprecipitated proteins, beads were washed in 0.1 N NaOH, incubated with Laemmli sample buffer and boiled for 5 minutes and centrifuged at 10,000 g for 5 minutes. Beads were discarded and supernatant analysed by gel electrophoresis. (8) 60

minutes at 37°C in conditioned medium obtained from BFA-treated acini. This was obtained from acini washed three times in the presence of 5 µM BFA then incubated for 60 minutes at 37°C in the presence of the drug. The conditioned medium was also analysed by immunoblotting for the presence of milk proteins or CD.

#### Detection of rPRL molecular forms and cathepsin D

Tissues and acini incubated with rPRL were washed three times and homogenised in 10 mM Hepes, 1 mM EDTA, 0.5 mM PMSF, 1 mM Mg (oAC)<sub>2</sub> buffer supplemented with a mixture of protease inhibitors (5 µl/ml) at 4°C. Following centrifugation at 800 g for 10 minutes at 4°C, post-nuclear supernatants were subjected to three cycles of freeze-thawing after addition of 1% IGEPAL CA-630. They were then spun at 259,000 g for 1 hour at 4°C and supernatants were analysed. To obtain the vesicular content, post-nuclear supernatants were centrifuged at 110,000 g for 60 minutes at 4°C. Pellets were resuspended in 1 mM EDTA, 10 mM Tris-buffer pH 8, subjected to three cycles of freeze-thawing after addition of 1% IGEPAL CA 630 and finally spun at 259,000 g for 1 hour at 4°C. Proteins obtained in the supernatant were precipitated by TCA 10% final. The pellet was washed by ether-ethanol 1:1 (v:v) and resuspended in 0.1 N NaOH then diluted in Laemmli sample buffer. All samples were resolved by SDS-PAGE in 13.5% acrylamide gel under reducing conditions. Electrotransfer and immunodetection were carried out as previously described (Lkhider et al., 1996). Rat PRL, CD and total milk proteins were detected with rabbit anti-rPRL antibody (1:3500), rabbit anti-CD antibody (1:300) and anti-mouse milk proteins antibody (1:5000), respectively, washed and then incubated with peroxidase-conjugated appropriate secondary antibody before being revealed by chemiluminescence. Representative immunoblotting (out of at least three) are shown.

#### Immunofluorescence

After incubation, acini were cytospun, fixed in 2% paraformaldehyde in 0.1 M sodium cacodylate, permeabilised with 0.1% Triton X-100 in phosphate buffered saline (PBS) and then sequentially incubated with 50 mM NH<sub>4</sub>Cl in PBS (45 minutes), 1% bovine serum albumin (BSA) in PBS (60 minutes) and primary antibodies (120 minutes). The antibodies were diluted in PBS (1% BSA) as follow: anti-mouse milk protein (1:1000), anti-rat CD (1:100), anti-rat TGN38 (1:10). Samples were washed and incubated with the appropriate fluorescence-conjugated secondary antibodies. Mammary tissues from lactating rats were fixed, frozen and cut as described (Seddiki et al., 2002). The sections were treated for immunofluorescence as described for acini. The histology of the mammary gland has already been described (Richert et al., 2000). To further ascertain the integrity of the tissue architecture, nuclei were stained with 4',6'-diamino-2-phenylindole (DAPI).

#### Scanning electron microscopy

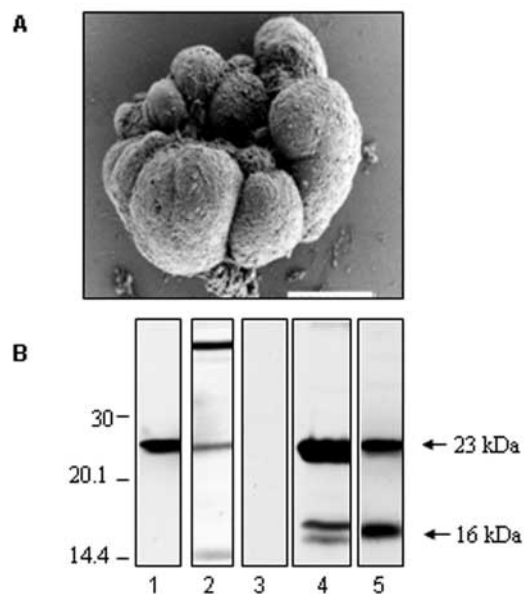
Acini were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate and 1% OsO<sub>4</sub> in the same buffer, dehydrated and critical-point-dried using freon. The samples were gold-metallised and observed on a Hitachi S450 (Elexience, Verrières-le-Buisson, France) scanning electron microscopy.

## Results

### Cleavage of rPRL is obtained in rat acini homogenates and in incubation media at physiological pH

When added to a 25,000 g membrane sediment of rat mammary tissue homogenate, rPRL is cleaved to release a 16 kDa PRL detectable under reducing conditions (Wong et al., 1986). This

fraction contains enzymes from the acini and also from cells present in the interstitial tissue (endothelial cells, blood cells, adipose cells). To verify whether MECs contain this processing ability, enzymatically dissociated acini depleted from stroma cells and strongly enriched in epithelial cells were prepared (Fig. 1A) and incubated with exogenous rPRL. As previously shown, a substantial amount of PRL was internalised in acini incubated in Hanks' medium in the presence of the hormone (Lkhider et al., 2001). When acini were incubated in the presence of 5 µg/ml rPRL for 15 to 30 minutes at 20°C, washed and then chased for 5 minutes at 37°C, endocytosed PRL was located in vesicles (Lkhider et al., 2001; Seddiki et al., 2002). In these conditions, immunoreactive rPRL forms, with relative molecular mass (*M<sub>r</sub>*) of 60,000, 23,000 and 14,000 were detectable in the fractionated vesicular content of acini (Fig. 1B, lane 2). We then checked by western blotting the molecular forms of PRL present in the different incubation media. Exogenous rPRL added to Hanks' medium incubated for 1



**Fig. 1.** Detection of rPRL cleaved forms in vesicular fractions and incubation media from acini. (A) Scanning electron micrograph of acinus obtained after enzymatic dissociation of lactating mammary tissue. The 3D organisation and the morphological integrity are well preserved. Bar, 13.8 µm. (B) Electrophoresis under reducing conditions and immunoblotting analysis of rPRL molecular forms detected in tissue and in incubation medium of acini incubated in the presence of rPRL. Incubation media and acini were treated for immunoblotting as described in Materials and Methods. Immunoblotting analysis of: rPRL forms in Hanks' medium, incubated for 60 minutes at 37°C in the presence of rPRL (lane 1); vesicular content, obtained as described in Materials and Methods, of acini incubated in the presence of 5 µg/ml rPRL for 15 minutes at 20°C, washed and then chased for 5 minutes at 37°C, a time interval corresponding to the presence of PRL inside vesicles (lane 2); medium from acini incubated without exogenous rPRL (lane 3); medium from acini incubated for 60 minutes at 37°C in the presence of 5 µg/ml rPRL (lane 4). The PRL forms generated *in vitro* by incubating 1.5 µg/ml rPRL in citrate-phosphate buffer pH 3.2 containing bovine cathepsin D (enzyme to protein ratio 1:200) are also shown (lane 5). Positions of the molecular mass markers (kDa) are indicated on the left.



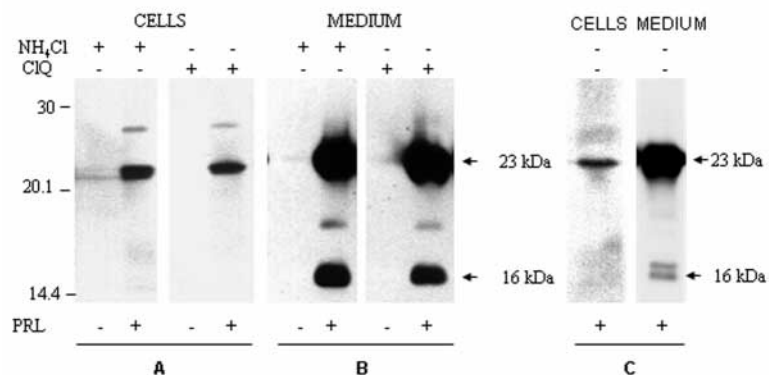
hour at 37°C was detectable as a single 23 kDa band (Fig. 1B, lane 1). No rPRL forms were detected in medium from acini that were not exposed to exogenous PRL, indicating that release of endogenous PRL was negligible (Fig. 1B, lane 3). By contrast, in medium from acini incubated for 1 hour at 37°C in the presence of exogenous rPRL, in addition to the 23 kDa band a doublet of approximately 16 kDa was clearly seen (Fig. 1B, lane 4). The latter showed a migratory rate similar to the migratory rate of the fragment generated by *in vitro* bovine CD-mediated proteolysis of rPRL at pH 3.2 (Fig. 1B, lane 5).

#### The 16 kDa PRL form is generated in the extracellular compartment, not within lysosomal acid organelles

Based on the finding that upon incubation with rPRL the 16 kDa PRL was detectable in the medium of MEC, not in the homogenate, we considered the possibility that exogenous rPRL was rapidly endocytosed, cleaved within intracellular acid compartments and the cleaved fragment rapidly released in the medium by exocytosis from endosomal-lysosomal organelles. Incubation with weak bases such as chloroquine and ammonium chloride is a valuable approach to assess the requirement for the endosomal-lysosomal hydrolysis of a substrate. Effects of these weak bases on the secretory activities of MECs have been previously described (Ollivier-Bousquet, 1980; Daudet et al., 1981). We incubated mammary fragments with 10  $\mu$ M chloroquine or 10 mM ammonium chloride in the absence or presence of rPRL and looked for the formation of the 16 kDa PRL in homogenate and media. When MECs were incubated with exogenous rPRL for 30 minutes in the presence or absence of either weak base, the 23 kDa form, but no immunoreactive cleaved PRL forms, was detectable in the homogenates (Fig. 2A,C). In the media of MECs incubated with the exogenous rPRL and in the presence or absence of ammonium chloride or chloroquine, in addition to the 23 kDa, the 16 kDa form was revealed (Fig. 2B,C). The intensity of the 16 kDa doublet appears enhanced in the presence of the weak bases compared with the doublet observed in the media of MECs incubated in Hanks' medium and a form with a  $M_r$  of about 18 kDa is also detectable. In a separate experiment, the addition of 10 mM  $\text{NH}_4\text{Cl}$  to the conditioned medium did not enhance the intensity of the doublet, thus excluding a direct effect of weak bases on the PRL processing enzyme (not shown). One possibility is that weak bases favour the release in the medium of factors that enhance the generation and/or stabilization of the PRL fragments. From these results the following conclusions can be drawn: (1) the added 23 kDa PRL is actively endocytosed by MECs, since no intracellular PRL forms are detectable in the absence of exogenously added rPRL; (2) no processed forms are detectable within the acini cells in which the lysosomal hydrolytic function is impaired; and (3) the 23 kDa can be cleaved directly in medium at physiological pH, regardless of the fact that lysosomal hydrolysis is impaired by a weak base.

To explain the presence of processed PRL forms in medium of acini incubated with exogenously added 23

kDa rPRL we considered the possibility that a proteolytic enzyme acting on the hormone at the pH of the medium was secreted by the MECs. To verify this hypothesis native 23 kDa PRL was incubated for 60 minutes at 37°C with the conditioned medium of acini that had been incubated under control condition. The western blotting analysis of the products obtained after such incubation revealed the appearance of a doublet positioned around 16 kDa (Fig. 3A) that resembled the position of the doublet previously shown in the incubation medium of acini exposed to rPRL (see Fig. 1B, lane 4). The migratory rate of the lower band of the doublet was similar to the migratory rate of the fragment generated by *in vitro* bovine CD-mediated proteolysis at pH 3.2 of rPRL (Fig. 3B). The rPRL-cleaving activity of the acini-conditioned medium was not apparent when incubation was performed at 4°C or when the acini-conditioned medium was heated at 100°C for 5 minutes prior to the incubation with rPRL (Fig. 3A). To confirm the rPRL-processing properties of the acini-conditioned medium we studied the time-dependent efficiency of the proteolytic process. The time-course analysis of the cleavage of rPRL by the conditioned medium revealed that the lower band of the doublet increased with time and that cleavage of the hormone reached near completion after 16 hours at 37°C (Fig. 3C). No spontaneous degradation was detectable after incubation of rPRL in non-conditioned medium, in the same conditions (Fig. 3D). It is notable that the aggregate big-PRL molecules progressively decreased and were almost undetectable at 16



**Fig. 2.** Effect of ammonium chloride and of chloroquine on rPRL cleavage in rat MECs. Electrophoresis under reducing conditions and immunoblotting analysis of rPRL forms detectable in mammary epithelial cells and in their media upon incubation with 23 kDa rPRL in the presence or absence of 10 mM ammonium chloride ( $\text{NH}_4\text{Cl}$ ) or 10  $\mu$ M chloroquine (ClQ) as indicated. Mammary fragments were preincubated for 30 minutes at 37°C in the presence or absence of the drug, then 5  $\mu$ g/ml rPRL was added or not for 30 minutes at 20°C in order to accumulate the hormone intracellularly and further incubated for 60 minutes at 37°C. Incubation media and fragments were treated for immunoblotting as described in Materials and Methods. (A) Immunoblotting analysis of rPRL forms in homogenates from tissue fragments (cells) incubated in the presence of weak bases. The 23 kDa form of rPRL was detectable in tissue incubated in the presence of exogenous PRL. (B) Immunoblotting analysis of rPRL forms in the incubation medium of tissues fragments (medium) incubated in the presence of weak bases. In addition to the 23 kDa form of rPRL, a form with an  $M_r$  of about 18 kDa and a much more abundant form with an  $M_r$  of 16 kDa were detectable. (C) Immunoblotting analysis of rPRL forms in homogenate and medium of tissue fragments incubated in Hanks' medium in the presence of exogenous rPRL. In tissue fragments (cells) the 23 kDa was detectable. In the medium, the 23 kDa and the 16 kDa forms were detectable. Positions of the molecular mass markers (kDa) are indicated on the left.

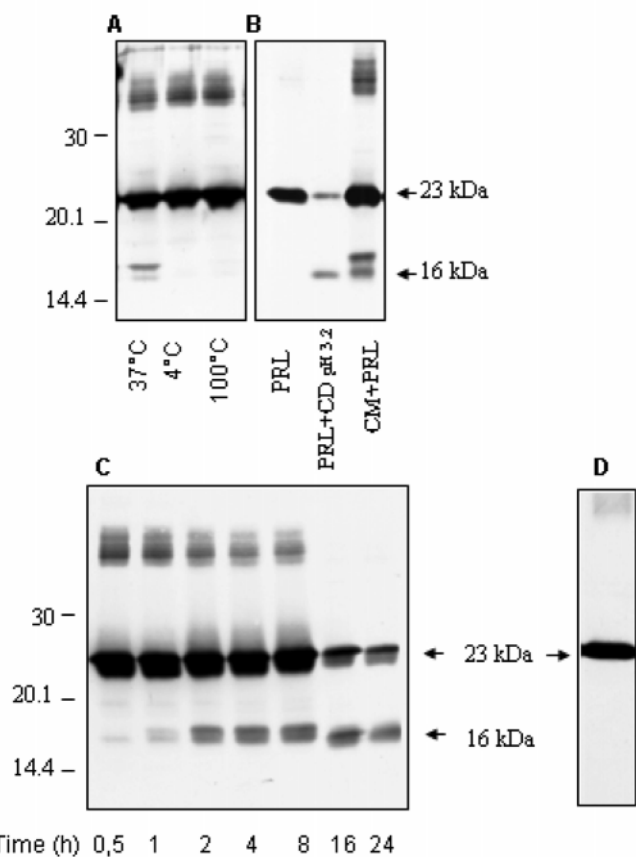
hours. It can be suggested that aggregates can form only in the presence of a certain amount of 23 kDa PRL. Moreover, it should be noted that with time only the lower band of the doublet migrating at 16 kDa accumulated in medium. From these results we conclude that the acini-conditioned medium contains an enzymatic activity that, at physiological pH, can cleave the native 23 kDa PRL into the 16 kDa PRL, which is detectable under reducing conditions in a time-dependent manner.

Brefeldin A does not preclude the accumulation of the PRL-converting enzyme in the acini-conditioned medium  
To determine whether the presence of the PRL-converting

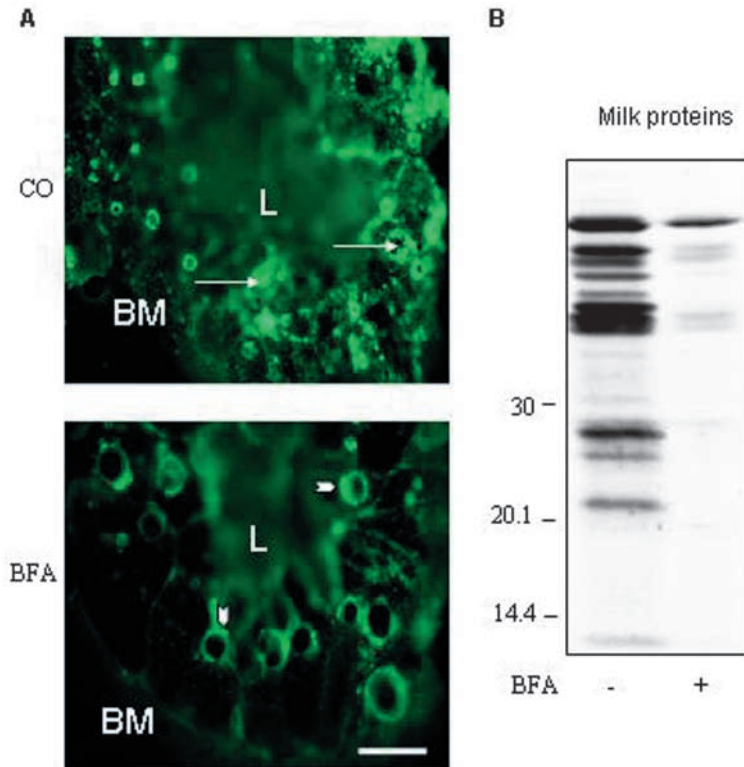
protease in the conditioned medium arose from a polarised secretion by MECs, the acini were incubated in the presence of BFA, a fungal toxin that is known to disrupt the Golgi apparatus organisation in MECs (Pauloin et al., 1997). BFA-treatment was associated with a strong decrease of milk proteins immunologically detectable in the secretory compartments of epithelial cells (Fig. 4A). Consistent with this morphological effect, BFA strongly inhibited the apical secretion of milk proteins (Fig. 4B). The next aim was to determine whether the PRL-cleaving activity was present in the medium of BFA-treated acini. As shown in Fig. 5, the cleavage of the 23 kDa PRL also occurred in the medium conditioned by acini in the presence of BFA, i.e. when apical secretion of glycoproteins from the Golgi apparatus was largely precluded.

The PRL-processing enzyme secreted by MECs identifies with lysosomal cathepsin D

In the search for the proteolytic enzyme that cleaves the 23 kDa PRL in the acini-conditioned medium, we considered as possible candidates thrombin and CD, two proteases that can cleave PRL in vitro at pH 7.4 and at pH 3.2, respectively. To assess the proteolytic effect of these two enzymes in physiological conditions, rPRL was first subjected to digestion by purified CD or thrombin at pH 7.4. To assess the specificity of their proteolysis, in parallel samples we included their respective inhibitor, i.e. pepstatin A (25  $\mu$ M) or hirudin (15 U/ml). Addition of CD generated a doublet migrating at approximately 16 kDa (Fig. 6A, lane 3). Pepstatin A strongly inhibited the cleaving activity of CD (Fig. 6A, lane 4). Addition of thrombin generated a 16 kDa fragment detectable as a single band that positioned between the higher and lower band of the doublet generated by CD (Fig. 6A, lane 2). Hirudin totally inhibited the thrombin-mediated PRL cleavage (Fig. 6A, lane 5) but did not affect the cleaving activity of CD generating the doublet (Fig. 6B, lane 2 vs lane 1). These results show that, at pH 7.4, both purified thrombin and CD are able to generate cleaved forms of rPRL, but the peptides generated have different sizes. We next addressed whether these enzymes are involved in the formation of the doublet obtained after incubation of rPRL in conditioned media. Hirudin added to the conditioned medium (Fig. 6C, lane 2) did not inhibit the cleaving activity of the medium involved in the generation of the doublet (Fig. 6C, lane 1). By contrast, pepstatin A strongly reduced the formation of the cleaved fragments (Fig. 6D, lane 2 vs lane 1). These results strongly suggested CD as the possible candidate of the cleaving activity in the incubation medium. To verify any involvement of CD in the physiological cleavage of rPRL we first assessed the presence of this protease in the acini culture medium. CD activity was detected in acini-conditioned medium by assaying the Pepstatin A-sensitive proteolysis of haemoglobin at pH 3.2 (not shown). Since under this assay condition the proCD undergoes self-autoactivation and contributes to the hydrolysis of the substrate we further investigated the molecular forms of CD secreted by MECs. As shown in Fig. 6E (lane 1) western blotting analysis of acini-conditioned medium revealed three major CD-related bands that correspond to the enzymatically inactive precursor of ~51 kDa, the single-chain mature form of ~44 kDa and the large chain of the double-chain mature form of ~30 kDa (the small chain of approximately 13 kDa probably ran out of the gel)



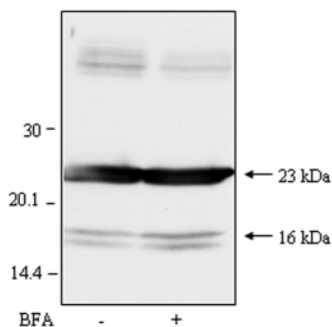
**Fig. 3.** Mammary acini-conditioned medium contains a PRL-cleaving activity. (A) Electrophoresis under reducing conditions and immunoblotting analysis of rPRL forms detectable in acini-conditioned medium upon incubation of rPRL. We used acini-conditioned medium, pre-heated to 100°C or not, in which 2.5  $\mu$ g/ml rPRL was incubated for 60 minutes at 37°C or at 4°C. The experiment reveals the formation of 16 kDa PRL in acini-conditioned medium at 37°C, but not in those conditions (4°C, pre-heating at 100°C) that are known to abolish enzymatic activities. (B) In vitro proteolysis of PRL. Immunoblotting analysis of rPRL after incubation for 60 minutes at 37°C in citrate buffer pH 7.2 (lane 1); in citrate buffer at pH 3.2 containing 0.1 U/ml CD (lane 2); or in conditioned medium at pH 7.4 (lane 3). The lower band of the doublet (lane 3) aligns with the 16 kDa form generated by total digestion of CD at pH 3.2 (lane 2). (C) Time course (from 0 minute to 24 hours) of PRL cleavage at 37°C in acini-conditioned medium. (D) Immunoblotting analysis of rPRL forms incubated in Hanks' medium for 6 hours at 37°C in the presence of rPRL. Positions of the molecular mass markers (kDa) are indicated on the left.



**Fig. 4.** Effect of BFA on milk protein secretion in MECs. Acini were incubated in the absence or presence of 5  $\mu$ M BFA for 60 minutes at 37°C, cytospun, fixed, permeabilised with Triton X-100 and treated for immunofluorescence. (A) In control acinus (CO) numerous secretory vesicles labelled with the RAM/MSP antibody are detectable in the cytoplasm (arrows). In the presence of BFA, cytoplasmic secretory vesicles decorated with the antibody RAM/MSP were no more detectable. It is notable that in BFA-treated cells huge lipid globules, whose periphery is strongly labelled with RAM/MSP antibody, accumulate at the apical region (arrowheads) (BM, basal membrane; L, lumen). Bar, 10  $\mu$ m. (B) Media from control and treated acini were analysed by immunoblotting to reveal the presence of milk proteins. In the control medium (lane -) immunoreactive bands correspond to the numerous milk proteins. The number of milk proteins found in the medium was drastically reduced in the presence of BFA (lane +).

(Demosz et al., 1999; Dragonetti et al., 2000). The latter two forms of CD are both resident within endosomes and lysosomes and are enzymatically active.

In order to have a clear-cut demonstration of the CD-mediated processing of rPRL, we prepared a conditioned medium immunodepleted of this protease. Acini-conditioned medium was extensively immunoprecipitated with specific



**Fig. 5.** BFA does not abrogate the PRL cleaving activity in acini-conditioned medium. Acini were incubated in the absence (lane -) or presence (lane +) of 5  $\mu$ M BFA for 60 minutes at 37°C as described in Materials and Methods and the conditioned media used for assaying the PRL-cleaving activity. To this end, 2.5  $\mu$ g/ml rPRL was added to the conditioned medium and after a 60 minute incubation at 37°C the samples were analysed by electrophoresis under reducing conditions and immunoblotting to reveal the PRL forms. The experiment demonstrates that the PRL-cleaving enzyme is present in the conditioned medium of BFA-treated acini. Positions of the molecular mass markers (kDa) are indicated on the left.

anti-CD antiserum and the resulting medium was analysed by western blotting. Fig. 6E (lane 2) confirms that almost all CD molecular forms previously present in the medium have been cleared by immunoprecipitation with anti-CD serum. The immunoprecipitate was recovered and analysed by gel electrophoresis followed by Coomassie Blue staining. This analysis suggested that the antiserum specifically precipitated CD, since no extra CD-unrelated molecular species were detectable in the precipitate (not shown), although the presence of tiny amounts of contaminants cannot be excluded. The 23 kDa PRL was then incubated for 60 minutes at 37°C either in native (containing CD) or in CD-immunodepleted conditioned medium at pH 7.4. In the absence of CD, the acini-conditioned medium lost its ability to generate the 16 kDa PRL form, an event that was associated with a higher accumulation of PRL aggregates (Fig. 6F). Finally, we wished to investigate the physiological site from where mature CD is secreted and operates the processing of PRL. We first look at the localisation of CD in the mammary tissue of lactating rat. Fig. 7 shows that CD is detectable in stroma cells, in MECs and in the lumen of acini. In MECs, spotted structures were detectable in the cytoplasm. It is of note that a strong labelling surrounding the acini revealed the presence of CD in the basal region of MECs. To verify whether CD located at the basal region of MEC can be released into the medium we treated the 3D-acini with BFA, which was shown to drastically impair the apical secretion of milk proteins, without affecting the capability of the medium to generate the 16 kDa PRL (see Figs 4 and 5). In previous work BFA was also shown to impair the secretion of proCD in cultured cells (Radons et al., 1990). The effect of BFA on the disassembly of the Golgi apparatus was confirmed by looking at the localisation of TGN38, an integral membrane protein of



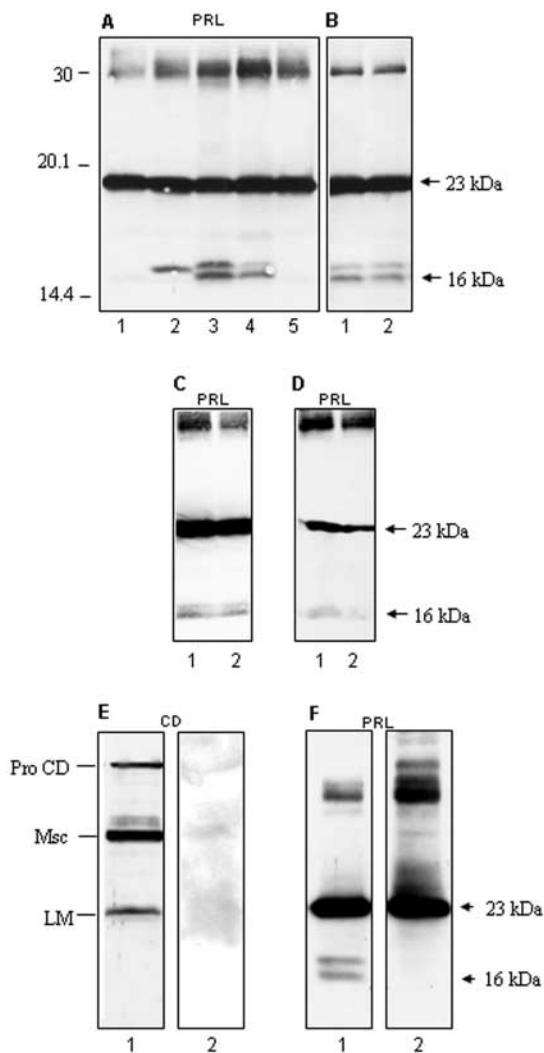
the TGN. In control acini, TGN38 was localised in a ring-shaped area between the nucleus and the apical membrane, while in BFA-treated acini TGN38 was weakly visible and showed a non-polarised location. The treatment with BFA did not affect the location of CD at the most basal region of acini cells (Fig. 8A). As shown in Fig. 8B, the drug did impair the secretion of CD to some extent, but some (25%) of the single-chain mature form and a lesser amount of the double-chain mature form of CD were still found in the medium. These data indicate that some CD can be exocytosed from endosomal-lysosomal compartments even when the organisation of the Golgi apparatus is disrupted and the apical secretion of glycoproteins practically abolished.

## Discussion

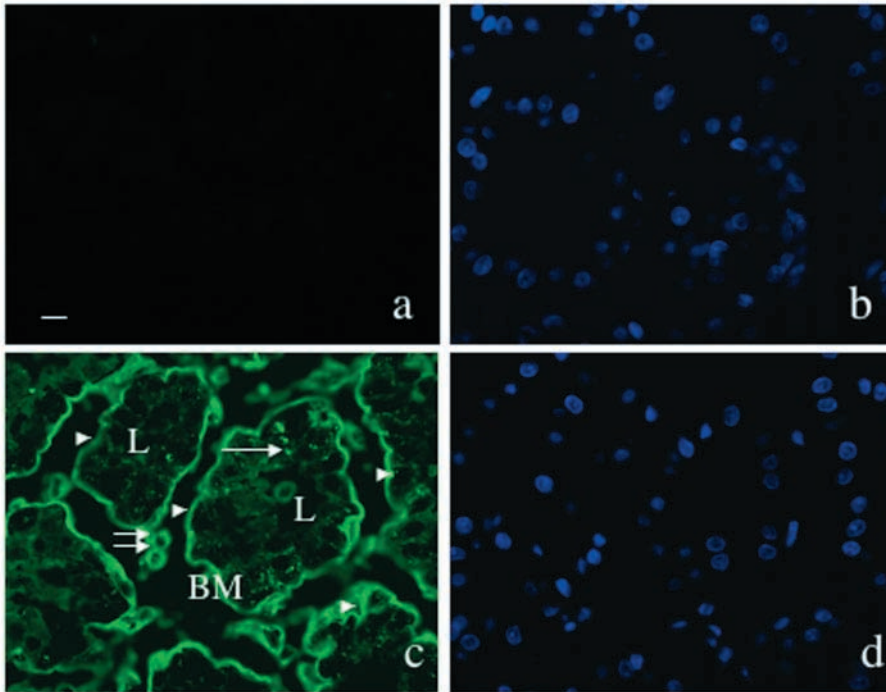
The main outcomes of the present work are the following. (1) The acini-conditioned medium contains the cleaving enzyme to effect at physiological pH the cleavage of intact rPRL, which yields under reducing conditions the 16 kDa PRL. (2) The MECs release immature and mature CD forms. (3) Depleting the acini-conditioned medium of CD abrogates its PRL cleaving activity. (4) BFA, while inhibiting the apical secretion

of milk proteins, does not preclude the secretion of the single-chain CD form, nor the processing of rPRL. These data suggest that for the processing of the 23 kDa PRL one specific CD molecular form is required and that this molecule is likely released at the basolateral site of acini. (5) Purified CD is able to effect the limited proteolysis of rPRL at pH 7.4, giving rise to the 16 kDa fragments.

Two results, reported in the present work, are in contrast with the established literature, i.e. the presence of mature forms of CD in the incubation medium of mammary acini and the enzymatic activity of CD at physiological pH. CD is an endosomal-lysosomal acid endopeptidase. It is synthesised as an inactive precursor that passes through the Golgi apparatus from where it is sorted by specific receptors to the endosome (Von Figura and Hasilik, 1986). In 2D *in vitro* cultures of mammalian cells a portion (depending on cell type and culture conditions) of the precursor is secreted in the medium (Hasilik and Neufeld, 1980; Isidoro et al., 1991; Isidoro et al., 1995a; Isidoro et al., 1995b; Isidoro et al., 1997a); this precursor can undergo auto-activation only if it is exposed at very acid pH. Within cellular acid compartments mature CD accomplishes either a limited or extensive proteolysis of various peptides, including pro-hormones and growth factors, thus contributing to their activation or inactivation (Berg et al., 1995). The finding of mature CD forms in the extracellular medium of MEC was somehow unexpected, since in several *in vitro* cell models, including mammary cancer cells that oversecrete CD, only proCD is found in medium (Capony et al., 1989; Isidoro



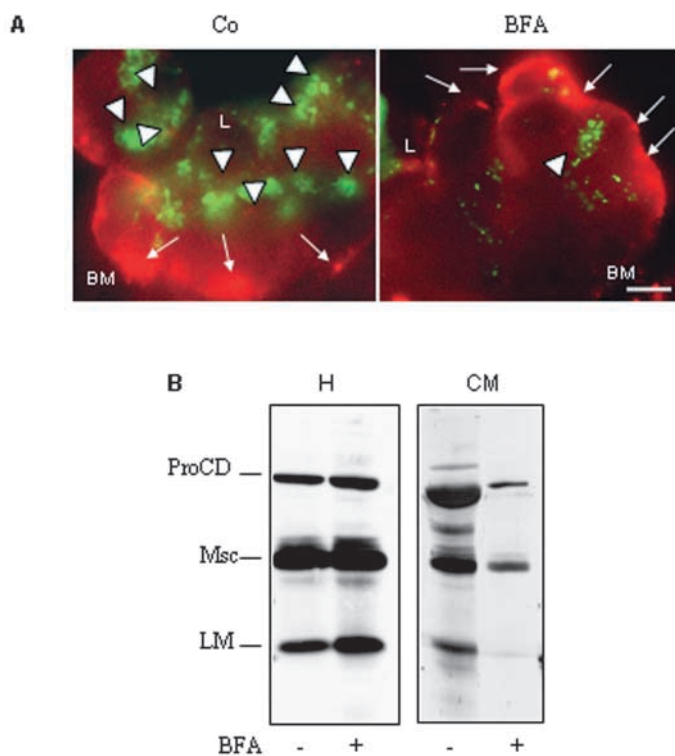
**Fig. 6.** Cathepsin D activity is required in the conditioned medium for an efficient cleavage of rPRL. (A) Proteolytic effect of purified thrombin and cathepsin D on rPRL. Immunoblotting analysis of rPRL forms when 1.5  $\mu\text{g/ml}$  rPRL was incubated in Tris-buffer pH 7.4 for 60 minutes at 37°C in the absence (lane 1) or presence of purified enzymes and their inhibitors: 12 U/ml thrombin (lane 2); 0.1 U/ml CD (lane 3); 0.1 U/ml CD and 25  $\mu\text{M}$  pepstatin A (lane 4); or 12 U/ml thrombin and 15 U/ml hirudin (lane 5). (B) Effect of hirudin on the cleaving activity of cathepsin D in Tris-buffer at pH 7.4 for 60 minutes at 37°C. Immunoblotting analysis of rPRL forms in the presence of 0.1 U/ml CD alone (lane 2) or with 15 U/ml hirudin (lane 1). (C) Effect of hirudin on the cleaving activity of the conditioned medium. Immunoblotting analysis of rPRL forms when 1  $\mu\text{g/ml}$  PRL was incubated for 60 minutes at 37°C in conditioned medium in the absence (lane 1) or presence of 15 U/ml hirudin. (lane 2). The doublet is detectable in both lanes 1 and 2. (D) Effect of pepstatin A on the cleaving activity of the conditioned medium. Immunoblotting analysis of rPRL forms when 1  $\mu\text{g/ml}$  rPRL was incubated for 60 minutes at 37°C in conditioned medium in the absence (lane 1) or presence of 10  $\mu\text{M}$  pepstatin A (lane 2). The doublet is hardly detectable in lane 2. (E) Acini-conditioned medium was depleted of CD by immunoprecipitation. In native medium (lane 1) the following rat CD molecular forms can be detected: ProCD, the 51 kDa precursor form; Msc, the 44 kDa single-chain mature form; LM, the 31 kDa large chain of the double-chain mature form. Almost all molecular forms of CD were cleared from the medium after immunoprecipitation with anti-CD serum (lane 2). (F) Immunoblotting analysis of rPRL molecular forms obtained by incubation for 60 minutes at 37°C of 2.5  $\mu\text{g/ml}$  rPRL in conditioned medium (lane 1) or CD-immunodepleted mammary acini-conditioned medium (lane 2). No cleaved forms of rPRL were detectable in the latter case. At the end of the incubation periods media were treated for immunoblotting as described in Materials and Methods.



**Fig. 7.** Immunofluorescence localisation of cathepsin D in mammary acini of lactating rat. Fragments of the mammary glands of rat were fixed and immunolabelled with anti-cathepsin D (1:100) and FITC-conjugated goat anti-rabbit IgG. (a) Control section treated as described with omission of the primary antibody. (b) DAPI staining of the control section. (c) Cathepsin D is located in cytoplasmic spots (arrow), in the lumen of the acini (L) and strongly accumulated in the basal region of the acini (arrowheads) in close proximity of the basal membrane (BM). Stroma cells are also stained (double arrow). (d) DAPI staining of the section showed in panel c. Bar, 10  $\mu$ m.

et al., 1997b; Rochefort and Liaudet-Coopman, 1999). We should add that, based on *in vitro* enzyme assays, mature cathepsin B and  $\beta$ -hexosaminidase, two other lysosomal enzymes, were also found in the acini-conditioned medium, thus confirming the occurrence of limited exocytosis from lysosomes (our unpublished observation). Consistently, in short-term primary culture of freshly isolated rat hepatocytes

we have indeed detected the presence of mature CD in the incubation medium (De Stephanis et al., 1997; Chiarpotto et al., 1999; Carini et al., 2004). Therefore, it is conceivable that in cells that maintain their polarisation the secretion of mature lysosomal enzyme is possible. With respect to the activity at physiological pH, it should be noted that *in vitro* CD has been shown to act at different pH, ranging from 2.8 to 6.0 depending on the substrate, thus arguing against any possible role in physiological fluid. *In vivo*, CD is believed to work only within the endosomal-lysosomal compartments at pH 3.0-5.0. Recently CD has been reported to translocate into the cytosol and to trigger the proteolytic activation of pro-apoptotic substrates (Bidère et al., 2003). Therefore, it is conceivable that the optimum pH *in vivo* varies with the substrate, perhaps also influenced by intermolecular interaction with physiological



**Fig. 8.** MECs secrete immature and mature forms of CD; BFA does not completely abolish this secretion. (A) Immunofluorescence localisation of CD and TGN38 (as a marker of Golgi apparatus) in acini incubated for 60 minutes at 37°C in the absence (control, Co) or the presence (BFA) of 5  $\mu$ M BFA. CD was detectable as red spots at the basal region of cells incubated in control medium (Co, arrows). A strong red labelling was also detectable in the basal region of acini incubated in the presence of BFA (arrows). In acini incubated in control medium, TGN38 (green) was located in the supranuclear region of cells (arrowheads) surrounding the aperture of the acinus which corresponds to the connection with the ductules (L). When acini were incubated in the presence of BFA, TGN38 was dispersed in the cells (arrowhead) confirming the disassembly effect of the toxin on the Golgi complex (BM, basal membrane; L, lumen). Bar, 10  $\mu$ m. (B) Immunoblotting analysis of CD in cell homogenates (H) and conditioned media (CM) from acini incubated as above. The CD molecular forms are indicated (P, proCD; Msc, mature single-chain; LM, large chain of the mature double-chain). The enzymatic active mature single-chain CD form is detectable in medium from BFA-treated acini.



activators. Indeed, the notion that CD is active only at very acid pH relies on the *in vitro* assay that uses haemoglobin as a substrate. This is a rather complex, multimeric high molecular weight protein, whose hydrolysis may necessitate a very acid pH. In principle, it cannot be excluded that for CD to effect a limited proteolysis on small substrates a very acid environment is not mandatory. Here we present compelling evidence showing the specific contribution of mature CD secreted by MECs in the cleavage of rPRL to generate at physiological pH a cleaved form that yields the 16 kDa PRL. Previous work showed that PRL incubated in the presence of mammary explants (at pH 7.4) was cleaved and generated a form of 14 or 16 kDa depending on the stage of lactation, whereas PRL incubated with mammary gland-conditioned medium at pH 7.4 was not cleaved (Baldocchi et al., 1992). Present results differ from this work by the demonstration of cleavage of PRL at physiological pH outside of the mammary cell. We demonstrated that thrombin is not involved in this proteolytic activity secreted by MEC. We also excluded any participation of cellular acidic compartments in the processing of PRL to 16 kDa PRL by employing ammonium chloride and chloroquine, two weak bases that are known to impair the acid-dependent function of lysosomal enzymes. Presently we cannot exclude that other factors are involved in a coordinated proteolytic cascade ending in the processing of PRL *in vivo*. Two cleavage sites for CD have been described on the molecule of PRL, one between Tyr145 and Leu146 and the other between Trp148 and Ser149 (Baldocchi et al., 1993), suggesting that the two molecular forms of the doublet might result from the cleavage by the same enzyme at these different sites. Whether indirect, by recruiting another enzyme, or direct, the action of CD on rPRL in the acini-conditioned medium at physiological pH appears evident from the data shown here. Moreover, *in vitro* experiments with purified CD confirmed the capability of this protease to cleave PRL at pH 7.4 to generate the 16 kDa fragments.

CD has been found under pro and mature forms in bovine and rat milk (Larsen and Petersen, 1995; Benes et al., 2002), but until now it has not been clearly established whether this enzyme in milk is secreted by the MECs or is released from blood and stroma cells. We show that mature CD forms are released from epithelial cells at both apical and basolateral sites. We could exclude that this secretion arose from death cells, since by morphology the integrity of acini looked preserved. Moreover, if CD in medium was the consequence of cell rupture, then the molecular forms of CD found in medium should reflect the same proportion as in the cells. This seems not to be the case, since the single-chain is secreted to a higher extent. Interestingly, this is the main molecular form of CD found in medium also when the apical secretion is practically abolished by BFA treatment. Under these conditions the transport of newly synthesised proteins to the basal region of MECs (Devinoy et al., 1995) might concur to the basolateral secretion of mature CD. This finding is in agreement with the morphological localisation of CD-containing organelles at the basolateral-most part of acini cells and consistently indicates that at least a portion of the mature CD found in the extracellular environment arises from the basolateral exocytosis of these organelles.

The present data are consistent with the extracellular cleavage of 23 kDa PRL by CD, in the basal region of the

mammary epithelium or in the interstitial region. It is not known whether the cleaved 23 kDa may yield a free N-terminal 16 kDa fragment in the conditions found in the basal region of the MECs. A 17 kDa PRL has been detected in human serum in nonreducing conditions (Warner et al., 1993), suggesting that PRL fragments may be released in physiological conditions. However, whether this fragment is C or N-terminal was not determined. It is known that 16 kDa PRL binds to specific receptor sites on capillary endothelial cells but not to PRL receptors localised on MECs. This might explain the dichotomous actions of native PRL and 16 kDa PRL. Intact PRL and 16 kDa PRL possess angiogenic and antiangiogenic activities, respectively (Struman et al., 1999; Corbacho et al., 2002). The regulation of the balance between these two molecular forms is therefore of great physiological importance for the control of the vascularisation in the mammary tissue. Depending on the enzymatic activity, more or less of one or the other form of prolactin will be produced within the interstitial space. The control of cleaving activity released by MECs, including the release of CD will prove to be an important field of investigation to understand the functioning of mammary cells.

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