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Prolactin Promotes the Secretion of Active Cathepsin D at the Basal Side of Rat Mammary Acini

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Cathepsin D (CD), a lysosomal aspartic protease present in mammary tissue and milk in various molecular forms, is also found in the incubation medium of mammary acini in molecular forms that are proteolytically active on prolactin at a physiological pH. Because prolactin controls the vesicular traffic in mammary cells, we studied, *in vivo* and *in vitro*, its effects on the polarized transport and secretion of various forms of CD in the rat mammary gland. CD accumulated in vesicles not involved in endocytosis in the basal region of cells. Prolactin increased this accumulation and the release of endosomal active single-chain CD at the basal side of acini. The CD-mediated proteolysis of prolactin, leading to the antiangiogenic 16-kDa form, at a physiological pH, was observed only in conditioned medium but not milk. These data support the novel concept that an active molecular form of CD, secreted at the basal side of the mammary epithelium, participates in processing blood-borne prolactin outside the cell, this polarized secretion being controlled by prolactin itself. (*Endocrinology* 149: 4095–4105, 2008)

PROLACTIN (PRL) EXERTS numerous effects on the function of the mammary gland (1), including a secretagogue effect (2, 3). This hormone provokes, *in vitro*, the increase of the secretion of milk components to the apical side of the mammary epithelium and the accumulation of a marker protein of the *trans*-Golgi network (TGN), TGN38, to the basal side of the epithelium (4). Other proteins are carried to the basal side of the mammary epithelium by a basolateral secretion pathway. For example, the lysosomal aspartic protease cathepsin D (CD) is released as a mature active enzyme into the incubation medium of *in vitro*-cultured mammary acini when the apical pathway of secretion is blocked (5).

CD is synthesized as a preproenzyme that undergoes co- and posttranslational processing during its transport along the exocytic pathway from the rough endoplasmic reticulum toward the endosomal-lysosomal compartment (6, 7). The precursor (pro-CD) is sorted at the TGN for secretion or lysosomal segregation. Two sequential proteolytic steps lead to the formation of the mature single-chain (Msc) and mature double-chain (Mdc) forms, which are both active and accumulate in endosomes and lysosomes, respectively. The Mdc is made up of a 34-kDa large chain [large mature (LM)] and a 13-kDa small chain (6, 8). CD synthesis and secretion have been extensively studied in breast cancer cells and tissues (see Ref. 9). Breast cancer cells overexpress CD and oversecrete the pro-CD form (10, 11). By contrast, few data exist on the synthesis and secretion of CD in highly polarized lactating mammary tissues. CD is present in the whey fraction of bovine and rat milk, mainly in the pro-CD form. Yet its origin in milk is unclear because the proenzyme can be secreted by different cell types (epithelial or stroma cells) (12, 13).

To what extent CD is also secreted at the basal side of mammary epithelial cells is not well known. Also unknown is the precise mechanism of the regulation of such transport to the basolateral side. We have shown that mature CD is secreted at the basal side by *in vitro*-cultured mammary acini and that this enzyme can ensure limited proteolysis of PRL at physiological pH to generate a 16-kDa PRL form (16K PRL) outside the cells. We also demonstrated that this form was different from the C-terminal 16-kDa fragment generated by thrombin and that the presence of CD in the medium is required for cleavage of the hormone (5). This form corresponds to the N-terminal 16-kDa PRL fragment (14–16) that has received considerable attention because of its potent inhibitory effect on angiogenesis *in vivo* and *in vitro* (17) and its importance in controlling microvascularization in different pathologies (18, 19).

PRL is also known to exert a secretagogue effect on the mammary epithelial cells (2, 3). We therefore hypothesized that in the mammary gland mature CD is secreted into the interstitial space after appropriate stimulation by PRL and so effecting the physiological proteolytic processing of plasma-borne PRL. This induced release of a protease responsible for its own processing into a biologically active fragment (the 16K PRL) would open new insights on the functions of this hormone and the physiology of the mammary gland. To test our hypothesis, we examined the secretion and activity of CD in rat milk and the conditioned medium of mammary acini in response to PRL stimulation. Here we provide evidence that CD is secreted in different molecular forms at the apical and ba-

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Abbreviations: BFA, Brefeldin A; Brat, bromocriptine-treated rat; CD, cathepsin D; conA, concanavalin A; FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; 16K PRL, 16-kDa PRL form; LM, large mature; Lrat, lipid-deprived rat; Mdc, mature double chain; MEC, mammary epithelial cell; Msc, mature single chain; PRL, prolactin; Pst, pepstatin; rPRL, rat PRL; TGN, *trans*-Golgi network.

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solateral sides of the polarized mammary epithelium and that PRL exerts a stimulatory effect in the intracellular transport and secretion of CD at the basal side of the epithelium.

Materials and Methods

Animals

Wistar rats at d 11–14 of lactation, weighing 180–250 g, were bred at Institut National de la Recherche Agronomique (Jouy-en-Josas). At the second day of lactation, the litter size was adjusted to 10 pups. At the 11th day of lactation, rats were treated with bromocriptine (a gift from Sandoz Pharmaceuticals, Hanover, NJ) for 48 h, as previously described, and called bromocriptine-treated rats (Brat) (20). Rats that received a diet containing no lipids since 50 consecutive generations were called lipid-deprived rats (Lrat) (21). All animal experimentation was conducted in accord with accepted standards of humane animal care. All ethical aspects of animal care complied with the relevant guidelines and licensing requirements laid down by the French Ministry of Agriculture. The protocol was approved by the local ethic committee (Ile de France Sud, France). Unless specified, all the experiments described below were repeated in at least three rats.

To collect milk, pups were removed 2 h before milking. Dams that were not used for morphological or biochemical studies were injected with 1 IU oxytocin (Syntocinon; Novartis Pharma, Rueil-Malmaison, France), anesthetized with 60 mg/kg sodium pentobarbital (Sanofi-Aventis, Paris, France) and then hand milked. The samples of milk were collected as described elsewhere (12), and the supernatant (skim milk) was obtained by centrifugation at 10,000 $\times$ *g* for 15 min. Anesthetized rats were killed by decapitation. Immediately after decapitation of the animals, the blood was collected and left for 30 min at room temperature and then spun at 2000 $\times$ *g* for 10 min to obtain the serum, which was stored at −80 C.

Preparation and incubation of mammary gland fragments and acini

Mammary tissues dissected free of connective and adipose tissues were cut into small fragments and incubated in Hanks' balanced salt solution (HBSS) (Life Technologies, Inc.-Life Technologies, Cergy-Pontoise, France) under an atmosphere of 95% O_2-5% CO_2 plus 0.2 g/liter sodium bicarbonate (pH 7.5) throughout the incubation period. Morphological and metabolic labeling studies demonstrated that under these experimental conditions, the secretory functions of the highly polarized mammary tissue (during the 3 h incubations) is optimal, and the expression of PRL receptors and the capacity to respond to the PRL stimulation of secretion are well preserved (2, 5, 22). Mammary fragments were incubated in the absence or presence of 1 μg/ml rat PRL (rPRL) (provided by Dr. A. F. Parlow, National Hormone and Pituitary Program, Torrance, CA) for 5 min then fixed and processed for the immunogold localization of CD. Enzymatically dissociated acini were prepared as previously described (5). Briefly, 5 mg of mammary tissue from the inguinal mammary glands, dissected free of connective and adipose tissue, were enzymatically dissociated and washed. Acini in suspension in fresh medium were evenly distributed for the different treatments. Acini were treated in the following ways: 1) incubated for 30 min at 4 C and then chased for 5 min at 37 C in the presence of fluorescein isothiocyanate (FITC)-concanavalin A (conA) or gold-labeled conA (Sigma, St. Louis, MO); 2) incubated for 30 min at 4 C in the presence of 1 mg/ml cationized ferritin (Sigma) and then chased for 1, 5, and 15 min at 37 C, in the absence or presence of 1 μg/ml rPRL; or 3) incubated for 15 min at 37 C in the absence or presence of 1 μg/ml rPRL. To evaluate the secretion of CD in the incubation medium, acini were preincubated in HBSS in the presence or absence of 5 μM brefeldin A (BFA) (Sigma) for 30 min at 37 C, extensively washed with fresh medium (to eliminate any CD that had accumulated in the cells before BFA took effect), and further incubated in the presence or absence of BFA and 1 μg/ml rPRL for 5 min at 37 C. The media were collected, filtered through 50-μm filters, and frozen.

After incubation, mammary fragments and acini were treated for morphological and immunocytochemical analysis. Some fragments were homogenized and fractionated.

Subcellular fractionation

Subcellular fractionation was performed after standard procedures (23), except that a discontinuous gradient of sucrose (15–50%) was used. Briefly, after incubation with or without PRL, the mammary acini were washed, resuspended in 250 mM sucrose/20 mM HEPES (pH 7.2) containing 1 mM EDTA and 1 mM phenyl-methanesulfonyl fluoride, and homogenized by 13 strokes in a glass/Teflon homogenizer. Unbroken cells and nuclei were pelleted by centrifugation at 300 $\times$ *g* for 10 min at 4 C. The postnuclear supernatant was layered on top of the sucrose gradient and subfractionated by ultracentrifugation at 68,000 $\times$ *g* for 35 min in a Beckman Ti-50 rotor. Finally, 24 fractions were recovered from the top (light density) to the bottom (heavy density) of the gradients. The density profile of the gradient was assessed using density-marker beads. The fractions enriched with endosomal-lysosomal organelles were identified with respect to their levels of β-hexosaminidase activity. Molecular forms of CD accumulating in these fractions were identified by immunoblotting.

Preparation of conditioned media

After enzymatic dissociation and washing, acini in suspension were evenly distributed and incubated in HBSS for 60 min at 37 C under an atmosphere of 95% O_2-5% CO_2. The conditioned media were obtained after filtration through 5-μm filters.

In vitro proteolysis of rat PRL

To study the processing of 23 kDa PRL in the various media (milk and conditioned medium), 1 μg/ml of rPRL was incubated at 37 C in the following media: 1) skim milk from lactating rat, Lrat, or Brat, each skim milk being diluted in citrate phosphate buffer (2:100) (pH 3.0) or Tris buffer (pH 6.8 and at 7.4, respectively), for 1 h; 2) citrate phosphate buffer (pH 3.0) or Tris buffer (pH 7.4) in the presence of bovine CD (enzyme to protein mass ratio of 1:200) for 4 h at 37 C; or 3) conditioned medium of mammary acini from control rats and Lrats for 1 h at 37 C.

Immunoblotting and immunoprecipitation

Samples were resolved by SDS-PAGE in 13.5% acrylamide gel under reducing conditions. Electrotransfer and immunodetection were carried out as previously described (5, 20). rPRL and CD were detected with rabbit anti-rPRL antibody provided by Dr A. F. Parlow (National Hormone and Pituitary Program) (1:3500) and rabbit anti-CD antibody produced in the laboratory of one of the authors (C.I.) (24) (1: 300), respectively, washed, and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody before revelation by chemiluminescence. Where indicated, before immunoblotting, CD was immunoprecipitated using pepstatin (Pst)-agarose beads, as previously described (8). Representative immunoblotting data (of a minimum of three experiments) are shown. Densitometric analysis were performed by analyzing immunoblots from five independent experiments corresponding to five different rats, using the Image J1.33u software (Wayne Rasband, National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/Ij/). The percentage of Msc CD form to the pro-CD form obtained from control and treated rats were paired for each rat. The resulting pairs of values were compared using Student's *t* test.

Immunofluorescence

Mammary fragments were fixed in a solution of 2% paraformaldehyde in PBS, treated as described (25), and incubated with 50 mM NH_4Cl in PBS (45 min), 1% BSA in PBS (60 min) and antirat CD (1:100) in PBS/1% BSA (120 min) and with the appropriate FITC-conjugated secondary antibody as previously described (5).

Immunogold electron microscopy

Mammary fragments and acini were fixed in a 2% paraformaldehyde/0.05% glutaraldehyde solution in PBS for 4 h; incubated with a 1% tannic acid solution in 0.1 M maleate buffer (pH 6), a 1% uranyl acetate solution in the same buffer; and then gradually dehydrated in ethanol (30% to 100%) and embedded in Unicryl. Sections were incubated in 50 mM NH_4Cl in PBS (20 min), 5% BSA in PBS, and 5% serum (from the

species in which the secondary antibody was raised) in PBS (30 min), 0.1% BSA-c in PBS (15 min), antirat CD antibody 1/1200 in 0.1% BSA-c (Aurion, Wageningen, The Netherlands) in PBS, 2 h or antirat PRL antibody 1:100 in 0.1% BSA-c in PBS, 2 h, washed with 0.1% BSA-c in PBS (30 min), incubated with a goat antirabbit IgG conjugated to 5 or 15 nm colloidal gold in the same buffer (30 min) and then in 5% uranyl acetate in distilled water (20 min). The sections were observed under an electron microscope (MIMA2 platform, Institut National de la Recherche Agronomique, Jouy-en-Josas; Philips, Eindhoven, The Netherlands).

Because anti-CD and anti-rPRL were both raised in rabbit, double immunolabeling of CD and rPRL with the 15 and 5-nm colloidal gold was performed using the two surfaces successively of a thin section, as described (26). The specificity of this procedure was tested during separate experiments in which the patterns of distribution of the gold particles were compared in sections labeled for PRL only or double labeled for both CD and PRL (not shown).

Quantitative evaluation of immunogold labeling

Mammary tissues were incubated in the absence or presence of PRL for 15 min. The experiment was repeated three times on tissues from three rats. Sections were treated for the immunogold localization of CD. The distribution of gold particles was counted on at least 20 micrographs per treatment per rat taken at random and under the same original magnification (×8000). The number of gold particles was counted in each cell compartment: 1) the cytoplasm including the rough endoplasmic reticulum and associated cytoplasmic region; (2) endosome-like vesicles; 3) multivesicular bodies; 4) lysosomes; 5) Golgi saccules; 6) the immature secretory vesicle; 7) mature secretory vesicles; 8) mitochondria; and 9) the nucleus. The percentage of gold particles was evaluated for each cell compartment. The values obtained from control and treated rats in each compartment were paired for each rat. The resulting pairs of values for three rats were compared using Student's *t* test.

Vesicles localized in a region of 4 μm in the basal part of cells (considered as a region rich in endocytic organelles in well-polarized mammary cells) were evaluated for the presence of CD by counting the 15-nm gold particles on electron micrographs taken at the original magnification of ×8000. Immunogold labeling in this region was quantified separately for the plasma membrane, vesicles positive for endocytosed proteins (cationized ferritin or PRL), and vesicles that did not label for such endocytosed proteins. Endosome-like vesicles were characterized on the basis of their homogenous electron-dense content, multivesicular bodies in terms of their content of small vesicles, and lysosomes on the basis of their heterogeneous content (accumulation of various remnants). Data were expressed as a percentage of gold particles in the different types of vesicles.

Results

PRL influences the transport and processing of CD in vitro

CD is ubiquitously expressed in tissues and is also present in blood and milk in different molecular forms (*i.e.* pro-CD, Msc, and Mdc). The origin of CD in these biological fluids has not been clearly established. We first compared the molecular forms of CD present in blood serum and milk with those secreted in a conditioned medium from lactating rat mammary acini (5). CD was precipitated from serum by means of Pst-agarose beads, which allows the efficient precipitation of all molecular forms of CD (8). Only the pro-CD form could be detected in blood serum (Fig. 1A, lane 1). In skim milk, the pro-CD form was detected along with a faint band showing an apparent molecular mass of 46 kDa (Fig. 1A, lane 6). Both these bands could be better appreciated after Pst precipitation (Fig. 1A, lane 8). As a comparison, the CD molecular forms present in the conditioned medium of cultured mammary cells (5) are also shown (Fig. 1A, lane 4). In the latter, the Msc form was mainly present as a band of electrophoretic mobility around 40 kDa. The LM form was present as a very faint band. We then considered whether the secretion of these CDs was under the control of physiological stimuli. PRL is strongly implicated in the intracellular transport mechanisms in mammary epithelial cells (2, 3). We investigated its role *in vivo* on the transport of CD into milk, in light of the fact that PRL does not exert its secretagogue effect in Lrat (21) and that treatment with bromocriptine, a dopaminergic agent, strongly reduces rat plasma PRL concentrations (20).

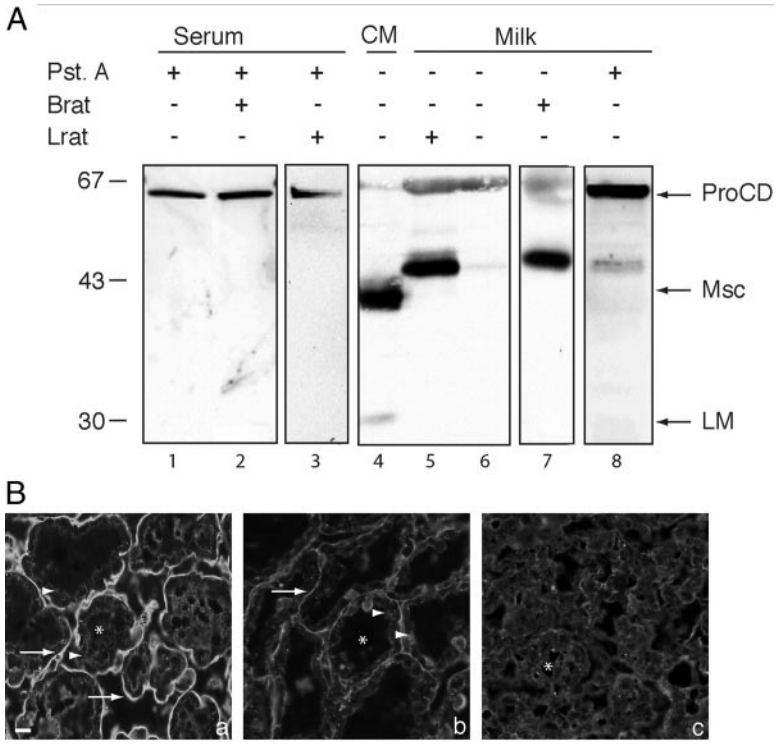

FIG. 1. Characterization of CD in serum, milk, conditioned medium, and mammary tissues, from Brat and Lrat. A, Proteins from serum, conditioned medium (CM), and skim milk were analyzed by SDS-PAGE and treated for immunoblotting, as described in *Materials and Methods*. Serum samples were precipitated by Pst-agarose (Pst.A) before analysis. Lane 1, Serum from lactating rat. Lane 2, Serum from Brat treated for 24 h. Lane 3, Serum from Lrat. Pro-CD was revealed in blood serum from control, Brats, and Lrats. Lane 4, Molecular forms of CD present in the conditioned medium (CM). Lanes 5–8, Molecular forms detected in skim milk. In skim milk from control rats, pro-CD was the principal CD form found in samples that had been previously precipitated (lane 8) or not (lane 6) by Pst-agarose. In skim milk from Lrat (lane 5) and Brat (lane 7), a CD-immunoreactive band with an apparent molecular mass of 46 kDa is prominent. This form was faintly detectable in skim milk from control rat (lanes 6 and 8). Position of the pro-CD, Msc, and LM is on the *right*. Position of the molecular mass markers (kilodaltons) on the *left*. B, Immunofluorescence localization of CD in mammary acini. a, Mammary acini of lactating rat. CD was strongly accumulated in the basal region of the acini (*arrows*) and was present as spots in the cytoplasm (*arrowhead*). b, After a 24-h bromocriptine-treatment, CD labeling in the basal region of acini was attenuated (*arrow*). CD was present as spots in the cytoplasm (*arrowheads*). c, After a 48-h bromocriptine treatment, CD labeling was diffuse in the cytoplasm and accumulated in the lumen. Acinar lumen (*asterisk*). *Bar*, 20 μm.

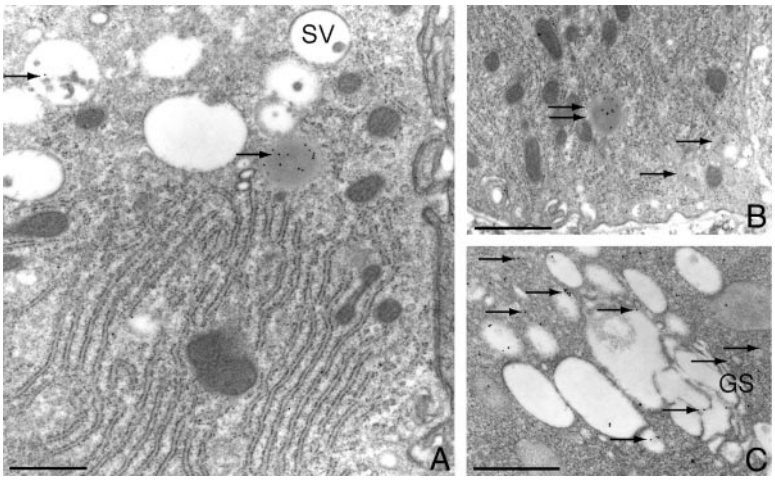

FIG. 2. Immunogold electron microscopy localization of CD in the mammary epithelial cells of lactating rat mammary tissue. A, CD was localized in different vesicles (*arrows*). SV, Secretory vesicle. B, In the basal region of the epithelium, dense vesicles (*double arrow*) and clear vesicles (*arrows*) were labeled. C, In the Golgi region, CD was present in Golgi saccules (GS) and frequently associated with coated pits and vesicles budding of the TGN (*arrows*). *Bar*, 1 μm.

We therefore analyzed the CD content in skim milk from rats in which the PRL effect (Lrat) or hormone status (Brat) had been modified *in vivo*. Only the pro-CD form was detected in blood serum from Brat and Lrat (Fig. 1A, lanes 2 and 3). Strikingly, in skim milk from Lrat (Fig. 1A, lane 5) and Brat (Fig. 1A, lane 7), the CD immunoreactive band of 46 kDa was the most prominent. Of note, the secretion of this 46-kDa CD form is dependent on PRL.

It is well known that the sugar composition of lysosomal enzyme results from the activity of glycan processing enzymes, whereas the proenzyme transits through the Golgi apparatus (27–30). Therefore, considering the specificity of the antibody used (8, 31, 32), it is likely that the 46-kDa CD form represents a highly complex type glycosylated Msc form. Indeed, the physiological status of Lrat and Brat is associated with dramatic changes in the Golgi apparatus organization (2), and this very likely has strong repercussions on its function as described in bovine mammary tissue (33, 34). CD localization was examined in control and Brat mammary tissues. In control tissues, CD was observed in the lumen of some acini as spots in the cytoplasm and surrounding the epithelium, which assumed a basal localization (Fig. 1Ba), in line with previous findings (5). Upon a 24-h treatment with bromocriptine of rats, CD staining in the basal region of the epithelium was strongly attenuated (Fig. 1Bb), and after a 48-h treatment, CD was no more detected in the basal region of the acini (Fig. 1Bc). In the latter condition, CD staining appeared rather diffuse in the cytoplasm and even more intense in the lumen of numerous acini, possibly associated with milk components including milk fat globule membrane (Fig. 1Bc). A similar pattern of CD staining was observed in tissues from Lrat (not shown).

Taken together, these observations are consistent with the hypothesis that *in vivo*, PRL controls the localization of CD and influences the processing and polarized sorting of CD in mammary epithelial tissue, thus accounting for the different molecular forms of CD present in blood serum and skim milk and in the conditioned medium of mammary acini. However, these observations did not enable investigation of the intracellular transport and secretion of CD and their control at a cellular level. To study these points, we shifted to a more cellular approach, *in vitro*, investigating the intracellular localization of CD in the absence or presence of PRL using immunogold electron microscopy.

Vesicles containing CD in the basal region of mammary epithelial cells are not involved in endocytosis

Immunogold localization of CD in lactating rat mammary epithelial cells (MECs) revealed its presence in the Golgi saccules and immature and mature secretory vesicles (Fig. 2, A–C), in line with its well-known trafficking within the exocytic pathway. CD accumulated in vesicles located in the Golgi region with a dense or more or less heterogeneous content characteristic of late endosomes and prelysosomes (Fig. 2A). Moreover, similar CD-positive vesicles were present in the basal region of MECs (Fig. 2B). To determine whether the latter were involved in endocytic events, a double localization of CD with different membrane markers of the endocytic pathway was performed. The mammary acini were incubated in the presence of FITC-conA and gold-labeled conA, a classical mannose-binding glycan (35) that binds to sugars on the cell membrane and is subsequently endocytosed. After 30 min of incubation at 4 C in the presence of FITC-con A, this molecule was found to accumulate at the extreme periphery of the cell (Fig. 3A). CD was also found to accumulate along the basal region of mammary cells (Fig. 3B). Double localization by immunogold electron microscopy confirmed that the two molecules were located in the basal region of cells but were not colocalized in the same vesicles (Fig. 3C).

Cationized ferritin that binds to negative charges on the surface of unfixed cells has been used as a marker of endocytosis (36). The distribution of ferritin particles makes it possible to follow the dynamic internalization of labeled membranes (3). Figure 4A shows that cationized ferritin was bound to basal portions of membrane and internalized in tubulovesicular compartments. In the presence of PRL, cationized ferritin accumulated in vesicles in the Golgi region (Fig. 4B). To determine whether the vesicles involved in the internalization of cationized ferritin also contained CD, acini were incubated for 30 min at 4 C in the presence of cationized ferritin and then chased for 1, 5, and 15 min at 37 C. The double localization of ferritin-labeled vesicles and vesicles

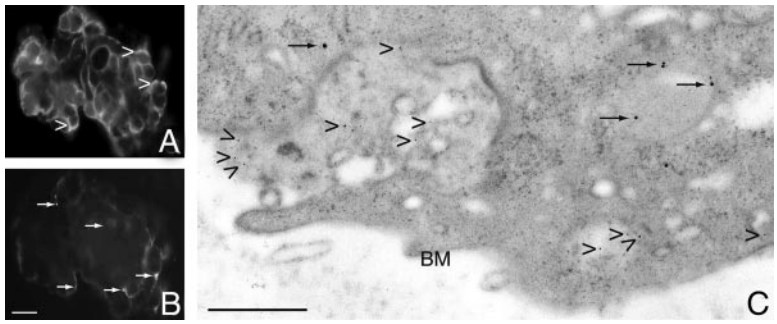

FIG. 3. ConA and CD do not localize in the same vesicles in the basal region of lactating rat mammary epithelial cells. Mammary acini were incubated for 30 min at 4 C and then chased for 5 min at 37 C in the presence of FITC-conjugated conA (*arrowheads*) (A) or gold-conjugated conA 5-nm gold particles (*arrowheads*) (C), fixed, and treated for either immunofluorescence detection of CD with antirat CD antibody and FITC-conjugated secondary antibody (*arrows*) (B) or for the immunogold electron microscopy detection of CD with antirat CD antibody and with a secondary antibody conjugated to 15 nm colloidal gold (*arrows*) (C). A, ConA strongly stained the periphery of cells. B, CD was observed as spots in the cytoplasm and at the basal region of cells. C, Immunoelectron microscopy shows that gold-conjugated conA (5 nm gold particles; *arrowhead*) are present in vesicles that differ from those labeled for CD (15 nm gold particles, *arrow*). BM, Basal membrane. *Bar* (A and B), 20 μm, (C), 200 nm.

containing CD detected by immunogold revealed the presence of different populations of vesicles in the basal region of MECs. Some of these were strongly labeled for ferritin alone, some for CD alone, and others for both (Fig. 4C). A quantitative evaluation of the percentage of gold particles associated or not with cationized ferritin in the various compartments in the third most basal region of MECs (plasma membrane, endosome-like vesicles, multivesicular bodies, and lysosomes) is shown in Fig. 4D. After incubation for 30 min at 4 C, cationized ferritin accumulated at the cell periphery and less than 1% of intracellular endosome-like vesicles contained ferritin. It was not possible to determine

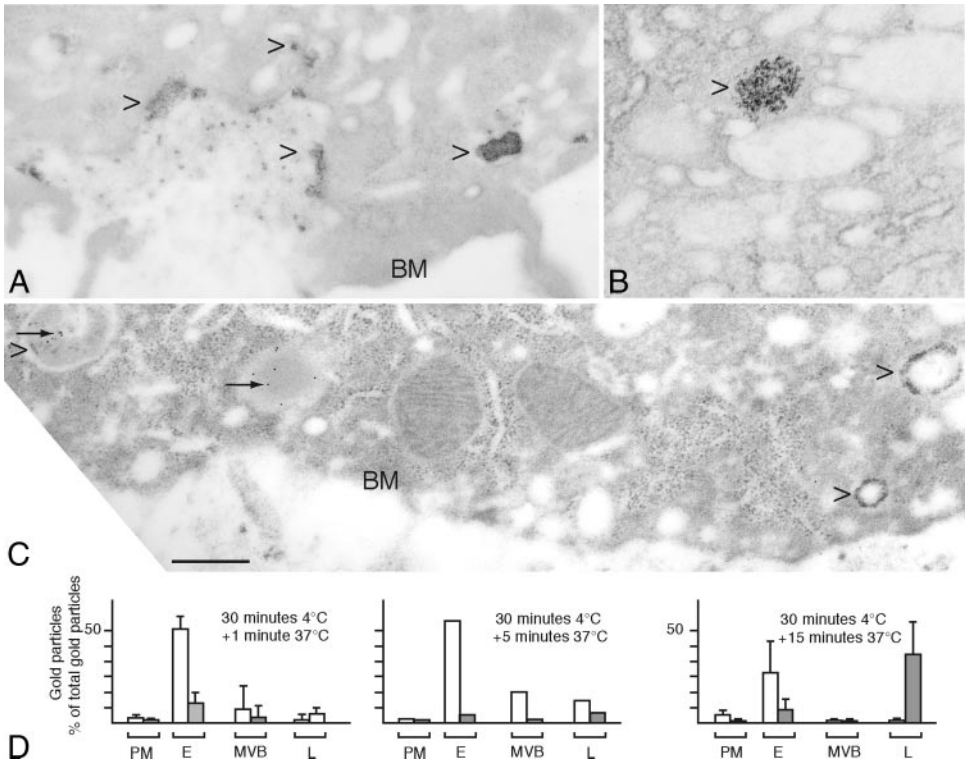

FIG. 4. Distribution of CD in vesicles labeled or not by cationized ferritin. Mammary acini were incubated for 30 min at 4 C in Hanks' medium containing 1 mg/ml cationized ferritin and then incubated at 37 C for 1 min in the absence (A) or presence of 1 μg/ml PRL (B). A, Cationized ferritin was associated with patches of the basal membrane (BM) and was detected in a vesicular tubular network close to the membrane (*arrowheads*). B, After incubation in the presence of PRL, cationized ferritin was frequently accumulated in cytoplasmic vesicles close to the Golgi apparatus (*arrowhead*). C, Double localization of CD and cationized ferritin in mammary acini incubated for 30 min at 4 C, chased, and then treated for the immunogold detection of CD, as described above. Cationized ferritin (*arrowheads*) and CD (*arrow*) were accumulated separately in different vesicles, whereas a lysosome contained both markers (*arrow* and *arrowhead*). *Bar*, 200 nm. D, Evolution of CD distribution in the different types of vesicles localized in the third basal part of mammary cells. The number of gold particles in percentage of the total gold particles was evaluated in vesicles not labeled with cationized ferritin (*white bar*) and in vesicles containing cationized ferritin (*gray bar*). PM, Plasma membrane; E, endosome-like vesicles; MVB, multivesicular bodies; L, lysosomes; *right* and *left panel*, means ± SEM, two rats; *central panel*, one rat.

whether the labeled structures were vesicles or membrane invaginations (quantitative evaluation not shown). The proportion of endosome-like vesicles containing CD not labeled by ferritin showed a highly statistically significant value, compared with endosome-like vesicles containing ferritin, after 1, 5, and 15 min at 37 C. The distribution of gold particles between endosomes with and without ferritin (16 *vs.* 84%) considerably deviated from the ratio 50:50 and the difference is considerably increased at 5 min, as shown by the highly significant test of heterogeneity [χ^2 with 2 $df = 14.8$ ($P < 0.001$)]. The percentage of mature lysosomes (recognizable by their content) that were positive for both CD and ferritin was increased after 15 min at 37 C due to an accumulation of internalized ferritin in lysosomes. To verify whether the vesicles containing CD were involved in receptor-associated endocytosis, a double localization of CD and PRL was performed. PRL arises from two sources in mammary epithelial cells. Plasma-borne PRL binds to PRL receptors present on the basal membrane and is internalized in endosomes (3). Endogenous PRL is also present in small quantities in all compartments of the secretory pathway (37). Immunogold labeling made it possible to detect PRL, yet it could not discriminate between endogenous PRL and plasma-borne PRL that had been internalized. To determine whether CD and PRL mixed in an endosomal compartment, a double immunogold localization of CD and PRL was carried out in mammary fragments. To increase the receptor-associated endocytosis of PRL, double immunogold localization was performed in fragments previously incubated in the presence of added rPRL.

In control fragments, CD and PRL were not localized in the same vesicles in the Golgi region (Fig. 5A). In the basal region, PRL was detected in endosomes that did not label for CD (Fig. 5B). By contrast, CD and PRL were frequently observed in the same regions of the extracellular matrix, suggesting that very small local modifications might facilitate an association (Fig. 5B). Interestingly, different types of vesicles were detected in acini incubated in the presence of added exogenous rPRL. Some vesicles were labeled only for CD, whereas small endosome-like vesicles were labeled only for

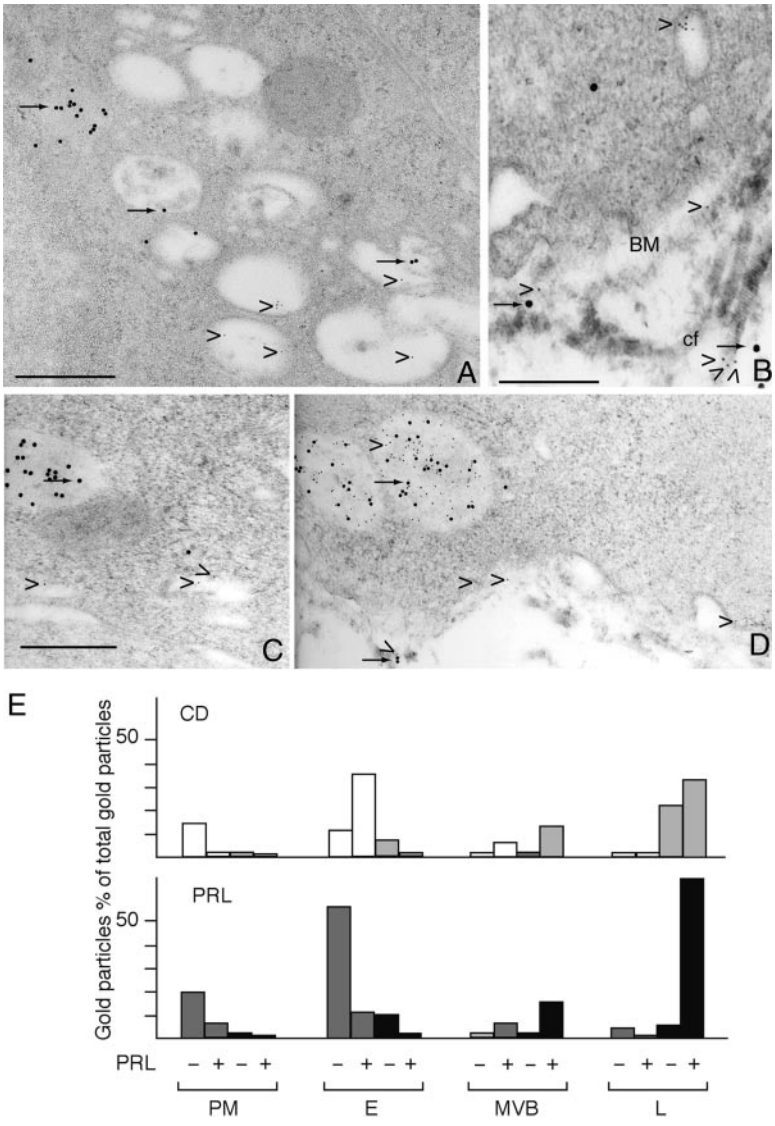

FIG. 5. Double localization of CD and PRL in mammary epithelial cells before (A and B) and after (C and D) incubation in the presence of PRL. A and B, Mammary tissue from lactating rat was fixed and treated for immunoelectron microscopy, first on one side of the grid with the anti-CD antibody, followed by 15 nm gold-conjugated antirabbit IgG, and then on the other side of the grid, with the anti-PRL antibody followed by the 5-nm gold-conjugated antirabbit IgG. A, PRL was detected in Golgi vesicles and secretory vesicles (*arrowheads*); CD was detected in dense vesicles and Golgi saccules (*arrows*). *Bar*, 200 nm. B, Endosomes close to the basal membrane (BM) contained PRL alone (*arrowhead*). CD (*arrows*) and PRL (*arrowheads*) were present in the extracellular matrix and associated with collagen filaments (*cf.*). *Bar*, 100 nm. C and D, Acini were incubated for 15 min in the presence of 1 μg/ml PRL, fixed, and treated for immunogold electron microscopy with anti-CD antibody and anti-PRL antibody as above. C, CD was accumulated in a vesicle close to the basal membrane (*arrow*) and rPRL was detected in different vesicles (*arrowheads*). D, CD and PRL were present in the extracellular matrix close to the basal membrane. An accumulation of PRL and CD were visible in lysosomes characterized by their heterogeneous content (*arrow* and *arrowhead*). *Bar*, 200 nm. E, Cellular distribution of CD (*upper panel*) and PRL (*lower panel*) in different cellular compartments of acini incubated in the presence or absence of PRL as described above. Results from one experiment representative of two experiments (20 micrographs were counted for each treatment). The gold particles (5 and 15 nm) corresponding to PRL and CD, respectively, were counted separately on the plasma membrane (PM) and the different vesicles. E, Endosome-like vesicles; MVB, multivesicular bodies; Lys, lysosomes. *White bars*, percentage of gold particles corresponding to CD in vesicles containing only CD. *Clear gray bars*, Percentage of gold particles corresponding to CD in vesicles containing both CD and PRL; *gray bars*, percentage of gold particles corresponding to PRL in vesicles containing only PRL; *black bars*, percentage of gold particles corresponding to PRL in vesicles containing both gold particles.

PRL (Fig. 5C). Other vesicles exhibiting the typical features of mature lysosomes (presence of cellular remnants) were labeled for both CD and PRL (Fig. 5D). A quantitative evaluation of the distribution of PRL and CD (Fig. 5E) revealed that very few endosomes contained both PRL and CD. In the cells incubated in the presence of PRL, the labeling of vesicles containing CD alone was increased, compared with the labeling in cells incubated in a control medium. Lysosomes contained both CD and PRL. It is noteworthy that after PRL stimulation there is a strong increase of colabeling of lysosomes by both CD and PRL. This enrichment of PRL in lysosomes is in accord with the internalization and sorting to the degradative pathway of an excess of exogenous PRL added to the medium.

These data strongly suggest that, in the lactating rat MECs, a population of vesicles containing CD located in the basal part of the cells do not serve endocytic functions (as defined by the endocytosis of conA, cationized ferritin, and PRL) but rather are part of the biosynthetic and secretory pathway.

PRL increases the basal localization and release of CD, in vitro

To investigate more precisely, and *in vitro*, the effects of PRL on CD localization, mammary acini were incubated in the absence or presence of PRL for 15 min. We first investigated whether PRL modified the CD molecular forms in cell fractions enriched in lysosomal β-hexosaminidase (fractions 10–18) (Fig. 6Aa). The fractions rich in β-hexosaminidase were positive for the presence of both Msc and Mdc forms (the large chain of the latter being clearly visible) of CD but not for pro-CD as expected. In control cells, the large chain of the Mdc was by far the most represented form [Fig. 6Ab (C)], whereas in cells incubated for 15 min in the presence of PRL, we observed an increase of the immunoreactive bands corresponding to the CD Msc form [Fig. 6Ab (PRL)]. We then analyzed the localization of CD in PRL-stimulated mammary acini. CD was localized in the RER, Golgi vesicles, saccules, secretory vesicles, and dense cytoplasmic vesicles (Fig. 6Ba), as was found in control cells (Fig. 2). In addition, a large number of vesicles localized near the basal region were very strongly labeled (Fig. 6Bb). A quantitative evaluation of the distribution of CD in different cell compartments was compared between control and PRL-stimulated tissues (Table 1). In the presence of PRL, the percentage of gold particles was markedly increased in endosome-like vesicles when compared with controls. This suggests that PRL actively deroutes CD-containing vesicles toward the basal region of the cell. Based on the findings reported in Figs. 3 and 5, these vesicles should not label for endocytic markers. Statistical comparison by χ^2 test shows that there is a nearly significant tendency for an increased accumulation of CD, upon a 15-min exposure to PRL in the endosome-like vesicles not labeled by ferritin ($P = 0.07$) (Fig. 6C).

Previous results had shown that CD was released in the incubation medium, even when apical secretion was impaired in the presence of BFA (5). We wonder whether PRL could, in addition to increasing the transport of vesicles containing CD to the basal region, also exert a stimulatory effect on the basal secretion of CD in the medium.

The presence of molecular forms of CD in the medium was confirmed by immunoblotting analysis of the incubation medium (Fig. 6Da). Two bands corresponding to pro-CD and Msc were detected. The large chain of the Mdc form was faintly visible, attesting that the lysosomal content was not released. After 15 min of incubation in the presence of PRL, the band corresponding to Msc was increased in the medium. BFA alone or associated with PRL also increased the intensity of the CD Msc form in the medium. A quantitative evaluation of the total CD secretion, analyzed by immunoblotting, was not possible because of the absence of a marker such as β-actin in the incubation medium of the lactating rat mammary acini and because of the stimulatory effect by PRL of the secretion of all the milk proteins. Thus, to analyze the effect of the different treatments, we compared the ratio of the Msc form to the pro-CD form, between the treatments. As shown in Fig. 6Db, PRL provoked a statistically significant increase of the release of the Msc form in the medium. In contrast, BFA and BFA plus PRL did not show any statistically significant effect. This result shows that BFA, which impairs the apical secretion of milk proteins in mammary cells (4), did not decrease the release of CD in the medium.

These data confirm that the active Msc form of CD is released at the basal side of mammary acini and indicate that PRL enhances this secretion.

CD secreted in milk and the conditioned medium from Lrat mammary acini is not proteolytically active on PRL at a physiological pH

We then questioned whether the CD found in milk was proteolytically active on PRL. The incubation of 23 kDa rPRL in skim milk from a lactating rat [Fig. 7A (C)] did not result in the processing of PRL at the three pH values examined (3, 6.8, and 7.4). Under the same conditions, a cleaving activity in milk from Lrat and Brat was observed only when incubation was performed at pH 3 [Fig. 7A (Lr; Lr+Br; C+Br)]. It should be noted that the milk from Lrat and Brat was shown to be enriched with a 46-kDa form of Msc. This form may be active only at an acidic pH. By comparison, and in agreement with previous findings (5), the cleaving activity of purified CD (in a 4 h incubation with PRL) reached its maximum at pH 3.0 [Fig. 7A (CD)]. Under the latter condition, the 23-kDa PRL form was strongly reduced, and very small fragments became apparent (not shown), suggesting that the 16-kDa form underwent extensive digestion. The cleaving activity was partial at pH 7.4 [Fig. 7A (CD)]. A clear cleaving activity releasing a doublet was observed after 1 h of incubation of PRL in a conditioned medium at pH 7.4 [Fig. 7A (CM)]. These results demonstrate that the Msc forms detected in milk and conditioned medium did not display a similar proteolytic activity.

Finally, we questioned whether the CD secreted *in vitro* by mammary acini from Lrat was proteolytically active on PRL. First, we analyzed the molecular forms of CD secreted by the mammary acini. Figure 7B shows that, whereas the pro-CD and Msc forms were present in the conditioned medium from control rat mammary acini (lane 1), only the pro-CD form was evidenced by Western blotting in the conditioned medium from Lrat mammary acini (lane 2). As expected [Ref.

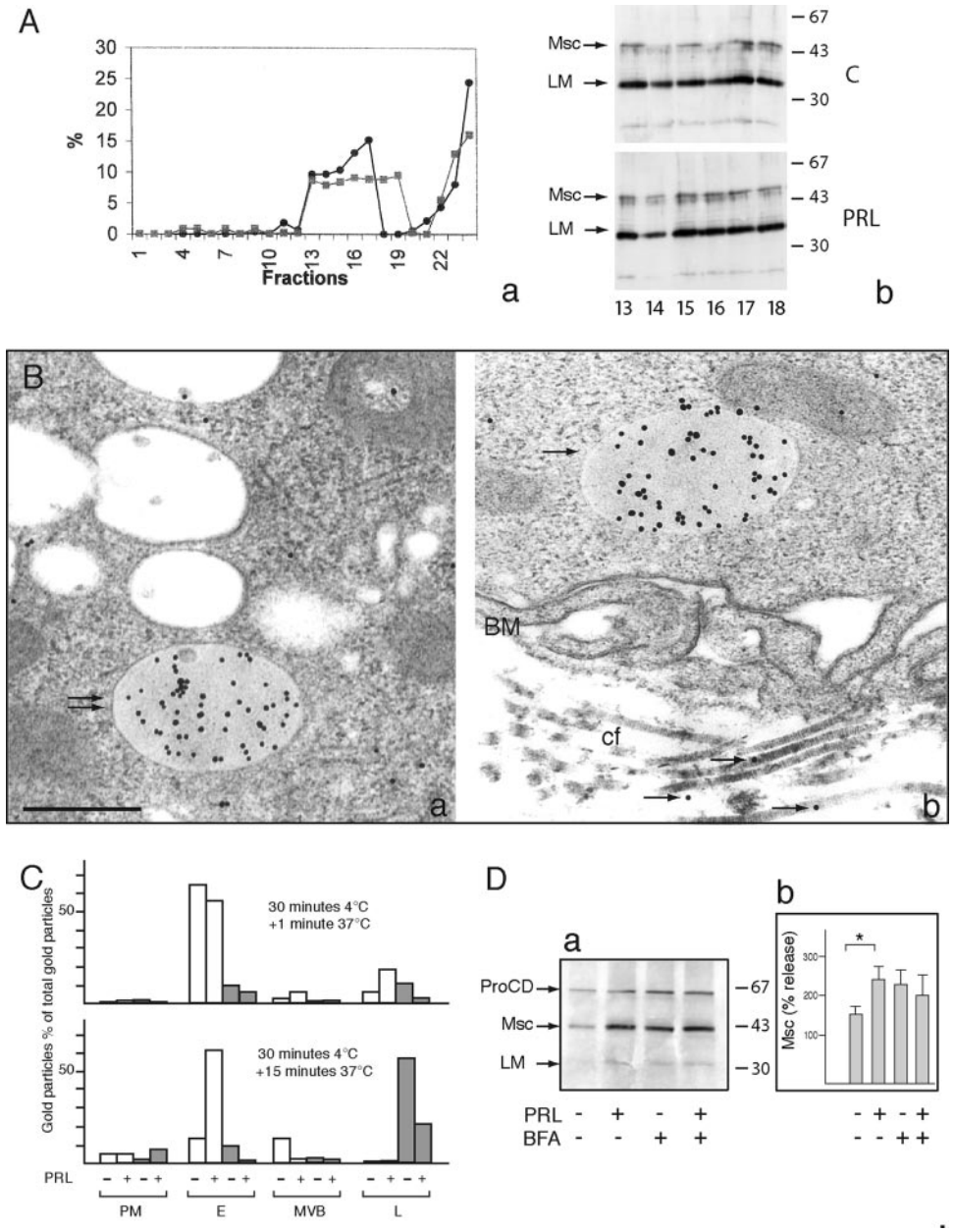

FIG. 6. *In vitro* effect of PRL on the intracellular transport and secretion of CD in lactating rat mammary epithelial cells. A, Mammary tissues were incubated for 15 min in the absence or presence of 1 μg/ml PRL, homogenized, and cell fractionated. The content of lysosomal β-hexosaminidase was evaluated in fractions from control mammary tissue (*gray squares*) and PRL-treated mammary tissues (*black spots*) (a). Molecular forms of CD accumulating in cell fractions enriched with lysosomal β-hexosaminidase (fractions 13–18) were identified by immunoblotting (b). The mature LM of the double chain of CD is the most represented form. In the presence of PRL, the immunoreactive band corresponding to the Msc CD form is more intense in these fractions. Position of the molecular mass markers (kilodaltons) is on the *right*. Result is representative of two experiments. B, Mammary tissues were incubated for 15 min in the absence or presence of 1 μg /ml PRL and then fixed and treated for immunogold electron microscopy with the anti-CD antibody, followed by 15 nm gold-conjugated antirabbit IgG. CD was strongly accumulated in endosome-like vesicles localized in the cytoplasm (a) and the basal region of mammary cells (b) (*arrows*). BM, Basal membrane. *Bar*, 200 nm. C, CD-labeled vesicles in the basal region that increased in the presence of PRL are not reached by the endocytotic marker, cationized ferritin. Mammary acini were incubated for 30 min at 4 C in the presence of 1 mg/ml cationized ferritin and then incubated for 1 and 15 min at 37 C in the absence or presence of 1 μ/ml PRL and then treated for immunogold, and the number of gold particles was counted, as described in Fig. 4. *White bars*, CD in vesicles not labeled by cationized ferritin. *Gray bars*, CD in vesicles containing cationized ferritin. Results from one experiment are representative of two experiments (15 micrographs were counted for each time period). D, PRL stimulates the secretion of the Msc form of CD in the incubation medium of mammary fragments. Immunoblotting analysis of the CD secreted in the incubation medium from lactating rat mammary acini preincubated in the presence or absence of BFA and then incubated in the presence of absence of PRL. The molecular forms of CD are indicated on the *left*. Position of the molecular mass markers (kilodaltons) is on the *right*. Densitometric analysis of the Msc released in the medium is shown (b). Values are expressed as the ratio of Msc to pro-CD (percent). The relative increase of Msc induced by PRL was statistically significant. *, $P < 0.05$. Five experiments were done (means $\pm$ SEM).

TABLE 1. Effect of PRL on the distribution of CD in mammary cells

	Control	Prolactin
Basal-lateral membrane	5 ± 1	5 ± 2
Endosome-like vesicles	10 ± 1	19 ± 3[a]
Multivesicular bodies + lysosomes	23 ± 6	26 ± 8
Golgi region (saccules + vesicles)	6 ± 3	6 ± 1
Secretory vesicles	6 ± 1	7 ± 2
Mitochondria	7 ± 2	7 ± 1
Nucleus	4 ± 3	4 ± 2
Cytoplasm	37 ± 4	25 ± 6

Mammary fragments were incubated in the absence or presence of 1 μg/ml prolactin fixed and treated for the immunogold localisation of CD, as described in *Materials and Methods*. Percentage of gold particles: three animals; means ± SEM.

[a] $P < 0.05$.

5 and Fig. 7A (CM)], after 1 h incubation of rPRL in the conditioned medium from control rat, a clear cleaving activity releasing a doublet was observed (Fig. 7B, lane 3). In contrast, in the conditioned medium from Lrat mammary acini, no cleaving activity was obvious (Fig. 7B, lane 4). These results strongly suggest that the CD forms released by the mammary tissue did not display the same proteolytic activity, depending on the *in vivo* physiological stage.

Discussion

The present results show that vesicles containing CD are carried to the basal region of acini under the control of PRL, both *in vivo* and *in vitro*. The release of a mature form of CD in the extracellular medium, at the basal side of the acini, is increased by PRL. In addition, this mature form of CD is proteolytically active to cleave PRL at physiological pH, unlike CD forms present in milk.

The intracellular localization of CD in MECs from lactating rats, as shown by immunogold labeling, reveals the presence of the enzyme in all cell compartments of the secretory pathway involved in protein synthesis and transport, including immature and mature secretory vesicles. This localization is consistent with the presence of pro-CD in milk (13), yet it raises questions concerning the mechanism of routing of this CD form to secretory vesicles. Does the pro-CD detected in milk result from the bulk flow of proteins escaping from the sorting toward endosomes and lysosomes?

The presence of different forms of CD in fluids representing apical (milk) and basal (conditioned medium in the presence of BFA) secretion can be explained by the intracellular sorting of the CD forms during maturation stages. Our data show that a faint CD-immunoreactive, 46-kDa form was present in milk from normal rats and that it was markedly increased in milk from Lrat and Brat. In the MECs of these animals, intracellular transport is markedly perturbed (2, 21). These disturbances may explain the presence of high quantities of the 46-kDa CD-related band, which probably reflects a highly glycosylated form. Until now, the role of CD forms in milk had not been clearly described. However, in CD-deficient transgenic mice, it was hypothesized that the CD received with milk ensured the viability of neonates until the fourth postnatal week (38).

The lactating mammary epithelial cell is a well-polarized cell whose intracellular secretory pathways are controlled by

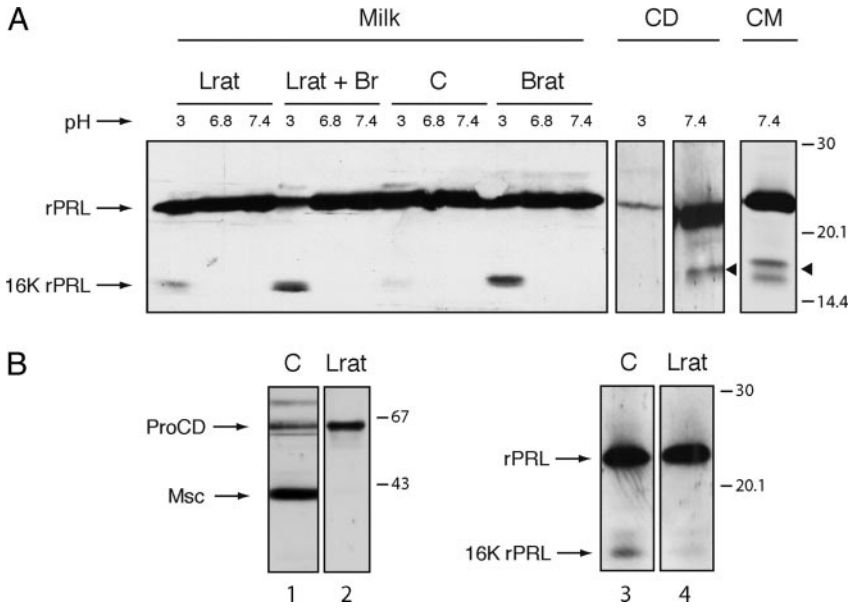

FIG. 7. PRL processing by milk and mammary conditioned medium. A, Rat PRL was incubated in skim milk from control (C), Lrat, Lrat treated by bromocriptine (Br), and Brat at various pH for 1 h at 37 C in citrate phosphate buffer and Tris-buffer containing CD at pH 3 and pH 7.4, respectively (CD), for 4 h and in a mammary acini conditioned medium at pH 7.4 for 1 h (CM). The presence of 23 kDa PRL (rPRL) and the 16K PRL fragment (16KrPRL) was detected by immunoblotting analysis using anti-PRL. In skim milk, PRL was processed only into 16K PRL at pH 3; in conditioned medium, PRL was processed into 16K PRL at pH 7.4. Position of the molecular mass markers is on the *right*. B, CD forms and PRL processing in the conditioned medium from control lactating rat (C) and Lrat. The presence of CD was detected in the conditioned medium of fragments from control rat and Lrat by immunoblotting analysis using anti-CD (lanes 1–2). In the conditioned medium from control rats, pro-CD and Msc were detected (lane 1). In the conditioned medium from Lrat, the pro-CD form was prominent (lane 2). Rat PRL was incubated in the conditioned medium from control rat (C) and Lrat (lanes 3–4). In the conditioned medium from control rat, the 16K PRL was apparent under a doublet (lane 3). In the conditioned medium from Lrat, 16K PRL was not visible (lane 4). Position of the molecular mass marker is on the *right*.

hormonal stimuli such as PRL and oxytocin (3, 39). The results presented here revealed CD-positive vesicles that do not serve endocytic functions accumulating in the basal region of cells. The number of such vesicles (and their CD content) increased after stimulation with PRL, strongly suggesting that their transport to the base of the cell is also regulated by this hormone. It has already been shown that polarized mammary epithelium carries different secretory proteins to the basal-lateral membrane (40). PRL provokes the basal accumulation of a TGN-associated protein (TGN38) (4). Our data (both *in vivo* in Brat and Lrat and *in vitro* in isolated acini) demonstrate that PRL has a positive influence on the basal accumulation of CD-containing vesicles. These results highlight a new feature of the hormonal regulation of MEC secretory processes by showing that the basal-lateral transport of vesicles and cargo is increased by PRL. The MEC thus appears to constitute a polarized model in which both apical and basal-lateral pathways are strictly controlled. CD-positive vesicles may represent a storage site for the enzyme that will be released on demand, this being analogous with similar findings in other cell types (41–45).

Taken together, our results support the notion that the intracellular transport and secretion of CD in MECs is tightly regulated by PRL and may in turn exert a proteolytic action on PRL in the extracellular fluid. They suggest a new working model of an autoregulation by PRL of its processing by CD (Fig. 8). The pituitary 23-kDa PRL binds to the prolactin receptors of the epithelial cells. Among the numerous biological effects of this hormone, intracellular transport associated with membrane trafficking is stimulated, leading to the secretagogue effect (2, 22). We demonstrated here that the hormone increases the transport of CD-containing vesicles to the basal region and stimulates the secretion of an active form of this protease into the basal extracellular medium. We have shown previously (5) and confirmed in the present work that Msc CD cleaves PRL at physiological pH. Thus, it can be hypothesized that *in vivo*, this proteolytically active Msc form, once secreted in the interstitial fluid, may cleave a part of the 23-kDa plasma-borne PRL and release locally a small quantity of 16K PRL. 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. However, the presence of this form in the serum of lactating women with peripartum cardiomyopathy has been described (19). The increase of this antiangiogenic form of PRL is a major contributor to pregnancy-induced cardiomyopathy, probably in relationship with a decrease of angiogenesis. In the mammary gland, the mammary vasculature grows during the first half of pregnancy and during the stages of cancer progression (46). These stages are characterized by an important angiogenesis. In contrast, microvasculature of lactating mammary gland is characterized by a nonproliferative, well-developed capillary network. Thus, the balance between the 23-kDa form of PRL (angiogenic form) and the 16K PRL (antiangiogenic form) (47) is in line with the control of the proliferative (pregnancy, cancer development) or nonproliferative (lactation) regulation of the microvasculature. Thus, during lactation, the high concentrations of plasma-borne PRL might regulate its own processing to an antiangiogenic form through the controlled release of active CD at the basal-lateral side of the mammary epithelium.

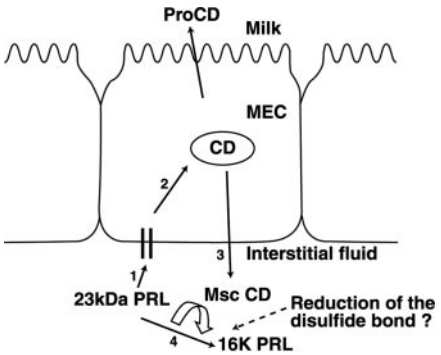

FIG. 8. A model of the regulation of transport of CD to the basal side of mammary acini. The plasma-borne PRL (23 kDa PRL) binds to the PRL receptor (*black boxes*) on the basolateral membrane of the MEC (1) and increases both the transport of CD-containing vesicles to the basal region of the cell (2) and the secretion of the active Msc form of CD into the extracellular medium at the basolateral side of the cell (3). Once secreted in the interstitial fluid, this proteolytically active Msc form may cleave a part of the 23-kDa plasma-borne PRL (4). Depending on a possible reduction of disulfide bond, a small quantity of 16K PRL might be released locally. The mechanisms of transport of pro-CD to the milk are unknown.

In conclusion, the results of this study provide evidence for a novel and previously uncharacterized aspect of PRL effect on the transport of different CD forms: through an effect on the secretory activity of the MEC, PRL may control the presence of a CD, which is proteolytically active on PRL, in the extracellular medium at the basal side of mammary acini.

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