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Establishment of plasma membrane polarity in mammary epithelial cells correlates with changes in prolactin trafficking and in annexin VI recruitment to membranes

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Abstract

Mammary epithelial cells (MEC) of lactating animals ferry large amounts of milk constituents in vesicular structures which have mostly been characterized by morphological approaches (Ollivier-Bousquet, 1998). Recently, we have shown that under conditions of lipid deprivation, perturbed prolactin traffic paralleled changes in the membrane phospholipid composition and in the cytosol versus membrane distribution of annexin VI (Ollivier-Bousquet et al., 1997). To obtain additional information on the membrane events involved in the vesicular transport of the hormone to the apical pole of the cell, we conducted a biochemical study on prolactin-containing vesicles in MEC at two different stages of differentiation. We first showed that MEC of pregnant and lactating rabbits exhibited membrane characteristics of non-polarized and polarized cells respectively, using annexin IV and the α-6 subunit of integrin as membrane markers. Incubation of both cell types with biotinylated prolactin for 1 h at 15°C, followed by a 10-min chase at 37°C revealed that prolactin transport was activated upon MEC membrane polarization. This was confirmed by subcellular fractionation of prolactin-containing vesicles on discontinuous density gradients. In non-polarized MEC, ^{125}I-prolactin was mainly recovered in gradient fractions enriched with endocytotic vesicles either after incubation at 15°C or after a 10-min chase at 37°C. In contrast, in polarized MEC, the hormone switched from endocytotic compartments to a fraction enriched in exocytotic clathrin-coated vesicles during the 10-min chase at 37°C. Association of annexin VI to prolactin carriers was next studied in both non-polarized and polarized cells. Membrane compartments collected at each gradient interface were solubilized under mild conditions by Triton X-100 (TX100) and the distribution of annexin VI in TX100-insoluble and TX100-soluble fractions was analyzed by Western blotting. Upon MEC polarization, the amount of annexin VI recovered in TX100-insoluble fractions changed. Quite interestingly, it increased in a membrane fraction enriched with endocytotic clathrin-coated vesicles, suggesting that annexin VI may act as a sorting signal in prolactin transport.

Keywords: Mammary cell; Polarized transport; Prolactin; Annexin VI

1. Introduction

A primary function of secretory and absorptive epithelial cells is to regulate the transit of selected

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molecules between the underlying tissue and the external lumen. In mammary epithelial cells (MEC) of lactating animals such vectorial transports mainly concern neosynthesized and blood-borne milk proteins which are ferried to the apical domain of mammary cells and secreted into the lumen. The impact of the polarized secretion in MEC is evidently important since milk provides the new-born with essential nutrients, as well as biologically active compounds such as growth factors and hormones. Besides the pioneer experiments of Keenan and collaborators on membrane compartments of mammary secretory cells reviewed in [3], there is an amazing lack of biochemical studies devoted to transcytosis carrier vesicles in MEC. Most information available on vesicular transport in these cells stems from morphological studies which detail the prolactin transit, showing that the hormone successively visits endocytotic vesicles, the Golgi apparatus and secretory vesicles carrying micelles of caseins [1]. In a long term program to determine the membrane signals participating in the vectorial transport of proteins from the maternal serum to the milk, we recently reported that in MEC of lactating rats fed with a lipid-deprived diet, prolactin trafficking is slowed down. This change in intracellular traffic parallels a modification in membrane phospholipid composition and in cytosol versus membrane localization of annexin VI [2]. Annexins belong to a multigene family of proteins characterized by their ability to bind negatively charged phospholipids in a Ca^{2+}-dependent manner. They contain a repeated conserved sequence of 70 amino acids termed the annexin core. This motif, which is involved in calcium and phospholipid binding, is repeated four times in all annexins except annexin VI in which it is repeated eight times. As a consequence, annexin VI (70 kDa) contains two distinct lipid-binding domains that could facilitate the cross-linking of membrane surfaces. Such an event has been reported to be a prerequisite for membrane fusion, a key step in directing vesicles in and out of cells [4]. This prompted us to propose that, in MEC, annexin VI may act as a membrane-sorting signal in the vectorial transport of prolactin.

Our current knowledge on vectorial transport, which stems from experiments in kidney cells, underlines the importance of specialization in both cellular architecture and plasma membrane composition [5]. We thus looked for MEC displaying different states of membrane polarization to obtain additional information on the membrane signals involved in prolactin transcytosis. Ultrastructural studies provide evidence that during pregnancy, differention events include (1) progressive increases in cytoplasmic volume, in the number and size of mitochondria and in the area of rough endoplasmic reticulum and Golgi membranes, (2) changes in cell morphology (shape, basal location of nucleus, Golgi size) [6]. In the present work, we have used membrane markers which specify basolateral and apical domains to show that MEC has the unique ability to switch from a non-polarized to a polarized state from pregnancy to lactation. Subcellular fractionation of prolactin-containing vesicles was carried out on both types of cells with particular attention to membrane compartments enriched in the rab5 protein, caveolin or the adaptors AP_2 and AP_1 which are specifically associated to the endocytotic and exocytotic clathrin-coated vesicles, respectively. The membrane characteristics of these compartments were probed using the solvation properties of annexin VI by Triton X-100 (TX100). Upon MEC membrane polarization, an increase in annexin VI recruitment to TX100-insoluble membrane domains was mainly observed in the fraction enriched with endocytotic clathrin-coated vesicles.

2. Materials and methods

2.1. Animals

Mammary glands were collected from New-Zealand female rabbits at day 20 of pregnancy and day 15 of lactation. All animal experiments including animal welfare and conditions for animal handling before slaughter were in accordance with the French guidelines (19 April 1988).

2.2. Materials

Materials were obtained from the following sources: Enhanced Chemi-Luminescence (ECL) kit from Amersham; ^2H_2O from Euriso-top (CEA, France); Texas-red extravidin and protease inhibitors (antipain, aprotinin, pepstatin, leupeptin, phenylmethyl-

sulfonylfluoride) from Sigma; nitrocellulose transfer membrane from Schleicher and Schuell; mouse monoclonal antibodies directed against the α-6 integrin subunit from TEBU; mouse monoclonal antibodies directed against annexin II and caveolin from Transduction Laboratories; mouse monoclonal antibodies directed against the p85 subunit of PI_3-kinase from UBI; mouse monoclonal antibodies directed against AP_2, AP_1 as well as peroxidase-labelled secondary antibodies from Sigma; sheep polyclonal antibodies directed against annexin VI were a gift from J.R. Dedman (Houston, TX, USA); rabbit polyclonal antibodies directed against the rab5 protein were a gift from P. Chavrier. Rat monoclonal antibodies directed against annexin IV were prepared as described elsewhere [7]. Iodination of ovine prolactin (kindly provided by Dr. Parlow, NIH, Bethesda, MD, USA) was performed by Dr G. Kann as described in [8]. Biotinylated prolactin was prepared as in [9].

2.3. Immunofluorescence

Dissected fragments of mammary gland were fixed immediately after slaughter with 2% paraformaldehyde in 0.1 M sodium cacodylate buffer for 2 h at 4°C, infused overnight with 40% sucrose in PBS and frozen in liquid nitrogen. Five μm thick sections were cut at −35°C with a 2800 Frigocut Reichert Jung (Leica, Rueil, France) and mounted on slides. After three washes with 50 mM NH_4Cl in PBS, sections were first incubated for 1 h with 1% bovine serum albumin in PBS (designated BS for blocking solution) then incubated for 1 h with primary antibodies against annexin IV (1/20) or the α-6 integrin subunit (1/2) in BS. After three washes with BS, sections were incubated for 30 min with the appropriate secondary antibodies diluted in BS. Sections were washed three times with BS, rinsed once with water, mounted in Mowiol 4–88 and examined using a Reichert-Jung Polyvar fluorescence microscope (Leica, Rueil, France).

2.4. Subcellular fractionation

Dissected fragments of rabbit mammary gland (about 100 g) were cleaned of connective tissues and homogenized in 200 ml cold MES buffer (100 mM MES, 2 mM EGTA, 0.5 mM $MgCl_2$, 0.3 mM NaN_3, pH 6.4) using a Waring blender (3×10 s bursts at low speed). The homogenate was centrifuged at 18 000×*g* for 20 min at 4°C. The supernatant was centrifuged at 100 000×*g* for 1 h at 4°C. The pellet was resuspended in MES buffer with a glass homogenizer fitted with a teflon pestle rotating at 3000 rev/min (three up-and-down strokes). Two rounds of low (18 000×*g*)/high speed (100 000×*g*) centrifugations were performed. The resulting 100 000×*g* pellet (crude vesicles) was resuspended in 2.5 ml MES buffer containing a cocktail of protease inhibitors. The mixture was loaded on a 5-step sucrose gradient prepared with 0%, 5%, 10%, 20%, 30% MES buffered sucrose solutions prepared in ^2H_2O. After centrifugation at 35 000×*g* for 4 h using a SW41 rotor, fractions were collected at the four interfaces (0–5%, 5–10%, 10–20%, 20–30%) whose densities were determined from the refractive index. Solubilization by 1% Triton X-100 in MES buffer was performed under mild conditions (4°C, 20 min) on gradient fractions previously subjected to two rounds of (−170°C; 37°C) freeze-thawing and pelleted at 100 000×*g* for 1 h.

2.5. Prolactin transport

Mammary fragments (10 to 40 mg) of pregnant and lactating rabbits were incubated for 1 h at 15°C in Hanks' balanced salt solution (HBSS) in the presence of biotinylated prolactin at 1 μg/ml. The non-interacting hormone was eliminated by three washes with cold HBSS. After a 10-min chase carried out at 37°C, the intracellular transport of prolactin was stopped at 4°C. Fragments were processed as for immunofluorescence (see above) and incubated for 20 min with extravidin (1/300). Sections were washed with BS, rinsed with water, mounted in Mowiol 4–88 and examined using a Reichert-Jung Polyvar fluorescence microscope (Leica, Rueil, France).

A second set of experiments was conducted under the same experimental conditions using ^{125}I-prolactin. Mammary fragments (100 to 400 mg) of pregnant and lactating rabbits were first incubated for 1 h at 15°C in HBSS in the presence of ^{125}I-prolactin. After elimination of the non-interacting material, 10-min chase periods were carried out at 37°C and

intracellular transport of prolactin was stopped at 4°C. Samples were homogenized and cell compartments were fractionated as described above. The prolactin content at each interface of the discontinuous gradient was quantified by counting radioactivity in an LKB gamma counter. Results are expressed as the percentage of the total labelling present at the four interfaces. The specificity of prolactin binding to its receptor was controlled by the addition of an excess of cold prolactin.

2.6. Western blotting

After determination of protein concentrations by the method of Peterson [10], membrane extracts were loaded onto slab gels (10% or 12% acrylamide, 1 mm thick). After SDS–PAGE, polypeptides were electrotransferred onto nitrocellulose for 1 h at 100 V (transfer buffer: Tris 25 mM, glycine 192 mM, 20% methanol, pH 8.3). The nitrocellulose sheet was incubated for 30 min at room temperature in PBS containing 5% fat-free dry milk powder and then incubated (separately) for 1 h at room temperature with antibodies directed against the rab5 protein (1/200), caveolin (1/200), the catalytic subunit of PI_3-kinase (1/500), the AP_2 or AP_1 adaptor (1/200), annexin II (1/2000) and annexin VI (1/2000). After three washes in PBS containing 0.3% Tween 20, immunoblots were incubated at room temperature for 1 h with the appropriate peroxidase-labelled secondary antibodies (1/2000). Detection was performed by Enhanced Chemi-Luminescence (ECL) and signals were quantified by densitometric scanning.

3. Results and discussion

3.1. Establishment of apical and basolateral membrane domains upon MEC differentiation

To assess whether MEC of animals at day 20 of pregnancy and at day 15 of lactation displayed a non-polarized or polarized membrane organization respectively, we conducted immunofluorescence studies using antibodies against markers of membrane domains.

The cellular localization of annexin IV which is expressed almost exclusively in epithelial cells [11] was first investigated. Fig. 1A shows that at day 20 of pregnancy, the annexin IV specific signal mainly delineate the contour of the MEC whereas at day 15 of lactation, the fluorescent signal is restricted to the basolateral domain of the cells (Fig. 1B). Such changes in the localization of the protein related to the stage of cell differentiation matches well with previous data obtained on hepatocytes and pancreatic acinar cells showing that in polarized cells, annexin IV is concentrated along the basolateral domain of the plasma membrane [7,12]. In poorly differentiated crypt cells of the intestine, an intracellular pool of annexin IV exists and labelling of the basolateral membrane appears to be lower than in mature villus cells. Likewise, the localization of annexin IV reflects the differences in the state of differentiation between the dome-forming and adherent cells from kidney tubules [13].

Integrins, a family of transmembrane heterodimer receptors composed of non-covalently associated α- and β-subunits, have been reported to define plasma membrane domains [14]. We observed that in the mammary tissue of pregnant animals, antibodies against the α-6 integrin subunit decorate the cell contour with the basal and lateral membrane domains brightly labelled (Fig. 1C). At day 15 of lactation, the labelling is restricted to the basal pole of the cell (Fig. 1D). In a recent study, it was shown using transgenic mice that altered localization of laminin and integrin reflects defects in cell polarization [15]. Moreover, Weaver et al. were able to reverse the malignant phenotype of human breast cancer cells by integrin blocking antibodies, indicating that a redistribution of α-6/β-4 integrin heterodimers plays a significant role in cell polarity [16].

The results of our experiments show that annexin IV and integrin α-6 are redistributed in specific membrane domains upon cell differentiation. We conclude that distinct basolateral and apical domains are established in MEC of lactating rabbits as a result of the transition from a non-polarized to a polarized state.

3.2. Vesicular transport of prolactin in non-polarized and polarized MEC

To determine whether, in MEC, polarized membrane architecture and cell trafficking are correlated,

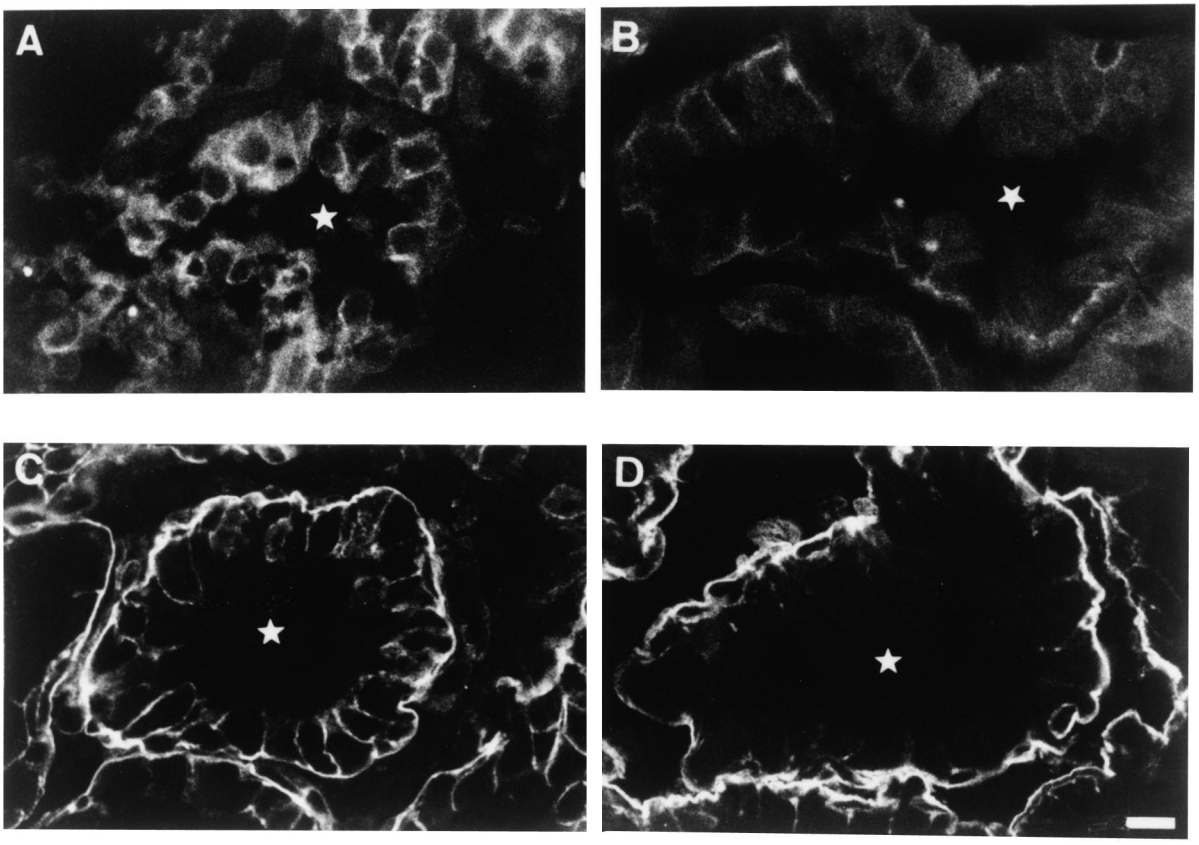

Fig. 1. Redistribution of annexin IV and α-6 integrin subunit in specific membrane domains upon MEC differentiation. Mammary tissue fragments were fixed in 2% paraformaldehyde and frozen. Thin sections were incubated with antibodies directed against annexin IV (A, B) and the α-6 integrin subunit (C, D). At day 20 of pregnancy annexin IV (A) and the α-6 integrin subunit (C) labelled the contour of the cell and the basolateral domain of the plasma membrane, respectively. At day 15 of lactation annexin IV is concentrated along the basolateral domain of the plasma membrane (B) whereas the α-6 integrin subunit is only detected at the basal pole of the cell (D). The asterisk indicates the lumen of the acini. Bar: 10 μm.

the route of prolactin was examined by incubating mammary fragments under experimental conditions favoring access either to early compartments of endocytosis (incubation at 15°C for 1 h) or to compartments of transcytosis (10-min chase at 37°C). The results obtained after incubation of MEC with biotinylated prolactin are shown in Fig. 2. At 15°C, a faint fluorescent signal is detected throughout the contour of non-polarized cells (Fig. 2A). After a 10-min chase at 37°C, the signal is uniformly distributed within the cells (Fig. 2C). After incubation of polarized MEC for 1 h at 15°C with biotinylated prolactin (Fig. 2B), punctiform fluorescent signals are detected at the basal pole of the cells. After a 10-min chase at 37°C (Fig. 2D), fluorescent spots are observed within the cell in the apical region, as previously reported [9]. The present comparative study clearly indicates that in MEC, the prolactin transport differs upon establishment of membrane polarization.

The labelling on the whole contour of the non-polarized cells (Fig. 2A) has to be noted. According to the results obtained in MDCK cells by van Meer and Simons [17], this observation may reflect a free lateral diffusion of the prolactin receptor along the plasma membrane due to 'leaky' tight junctions. As a matter of fact, preliminary experiments conducted with antibodies directed against ZO-1, a protein associated to the tight junctions, reveal a disorganized punctiform signal in non-polarized MEC whereas an organized continuous labelling is observed in polarized MEC (data not shown).

To obtain quantitative data, additional experiments were performed using ^{125}I-prolactin. Fig. 3

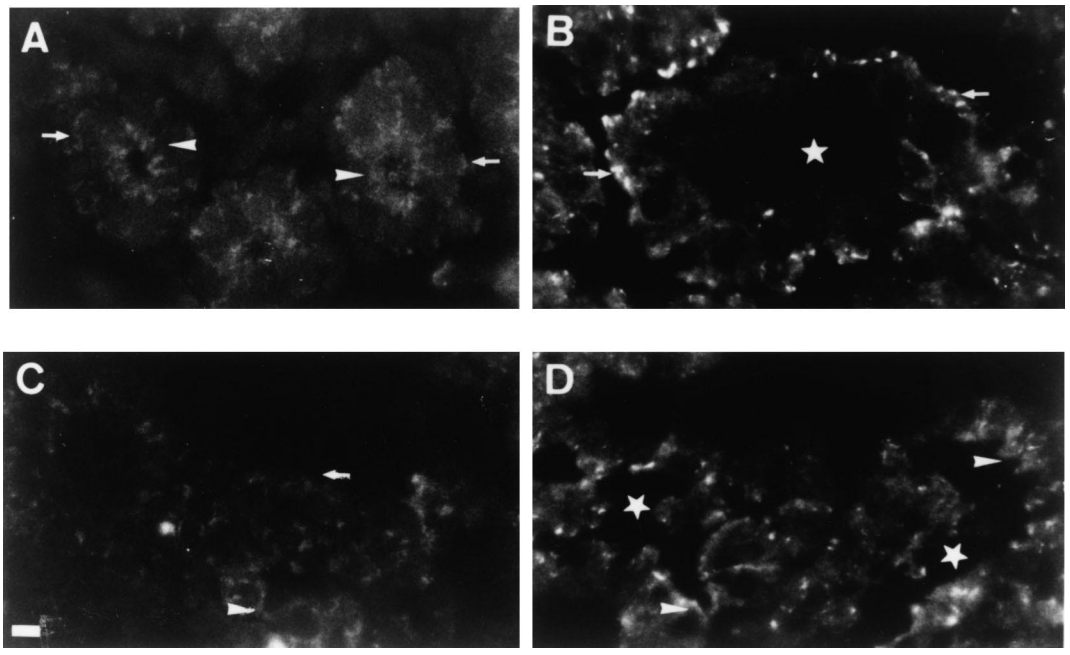

Fig. 2. Biotinylated prolactin trafficking differs in MEC of pregnant and lactating rabbits. Experiments were performed on fragments of mammary tissue from pregnant (A, C) and lactating (B, D) rabbits. The fragments were incubated with biotinylated prolactin at 15°C for 1 h. After extensive washing with HBSS at 15°C, followed or not by a 10-min chase at 37°C, the fragments were fixed in 2% paraformaldehyde and frozen. Sections were incubated with extravidin conjugated to Texas-red (TRITC) (1/300). In the absence of chase, the hormone is detected throughout the plasma membrane of MEC of pregnant rabbits (A) whereas in MEC of lactating rabbits the fluorescent prolactin concentrates at the basal pole of the cell (B). After a 10-min chase at 37°C, the signal is uniformly distributed in MEC of pregnant rabbits (C) whereas the signal is mostly detected in the apical region of MEC of lactating rabbits (D). Arrows and arrowheads point to the basal and the apical pole of MEC, respectively. The asterisk indicates the lumen of the acini. Bar: 10 μm.

(A,B) shows that, at 15°C, 45% of the radioactivity associated to prolactin accumulates in the membrane compartment of lowest density (interface 1) in both non-polarized and polarized MEC. In each of the three other compartments, the percentage of radioactive material never exceeded 25% in both cell types. This indicates that, at low temperature, despite the changes in the organization of the MEC plasma membrane, the hormone binds its receptor and accumulates in a target compartment of low density. As expected from previous data on the occurrence of prolactin receptors during mammary gland differentiation [18], lower amounts of radioactive material were systematically collected in gradients loaded with mammary membranes from non-polarized cells compared to polarized cells (2048 cpm versus 7852 cpm in a representative experiment). After a 10-min chase at 37°C, in non-polarized cells, the protein was still mainly detected in membrane compartments of low density (interface 1) (Fig. 3C). Under these experimental conditions, in polarized cells, the amount of labelled prolactin decreases in the membrane fraction of lowest density (interface 1), but increases significantly in the compartment of highest density (interface 4) (Fig. 3D) proving that polarized membrane architecture modulates the prolactin transcytosis in MEC.

To characterize the cell organelles carrying the hormone, Western blot analysis was performed using antibodies directed against the rab5 protein, caveolin, PI_3-kinase and the AP_2 and AP_1 adaptors. Fig. 4 shows representative data obtained with the different antibodies. The gradient profiles derived from the quantitative analysis of the ECL signals obtained with each collected fraction, are displayed in Fig. 5. The rab5 protein, reported to be associated with early endosomes in MDCK cells [19], is largely present in low density fractions (interfaces 1 and 2) isolated from crude vesicles of non-polarized as well as polarized MEC. Caveolins are a family of cyto-

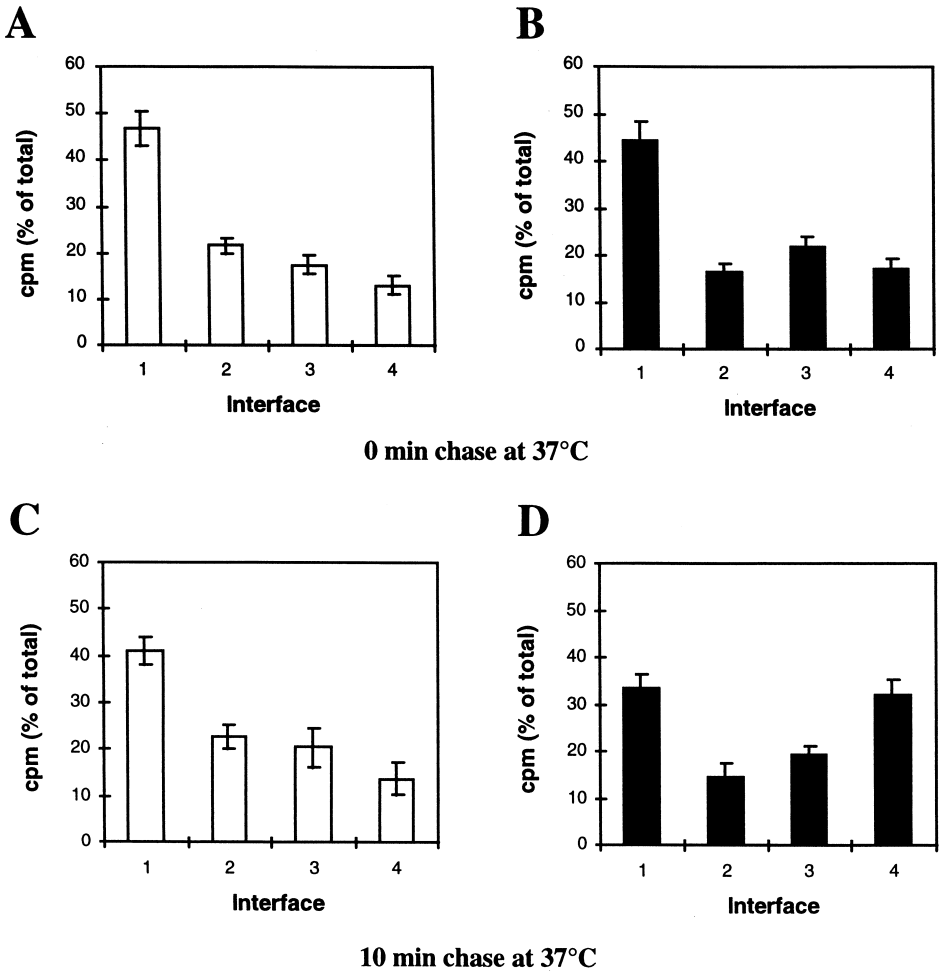

Fig. 3. ^{125}I-prolactin trafficking differs in MEC of pregnant and lactating rabbits. Experiments were performed on fragments of mammary tissue from pregnant (A, C) and lactating (B, D) rabbits. The fragments were incubated with ^{125}I-prolactin at 15°C for 1 h. Extensive washing with HBSS at 15°C was followed or not by a 10-min chase at 37°C. The preparation of crude vesicles and their fractionation on 5-step discontinuous sucrose density gradients were carried out as described in Section 2. Each bar represents the amount of radioactivity (expressed as the percentage of the total radioactivity) determined in each collected fraction of the 5-step gradients. Interface 1: $d=1.119$ g/ml; interface 2: $d=1.132$ g/ml; interface 3: $d=1.152$ g/ml; interface 4: $d=1.182$ g/ml.

plasmic membrane-anchored scaffolding proteins that help to sculpt caveolae membranes. Electron microscopic data showed that these vesicles are generated from invaginated pits and have a uniform diameter of 50 nm. They are involved in the transcytosis of macromolecules such as insulin, albumin and the HDL receptor and mediate scavenger endocytosis [20]. Fig. 5 shows that caveolin is mainly detected in the fraction of lowest density (interface 1). Although we cannot yet discriminate between endosomes and caveolae, the demonstration of the presence of caveolin in MEC is quite interesting. Indeed recent morphological and biochemical studies have brought evidence that growth hormone (GH), whose receptor shares structural and signaling homologies with the prolactin receptor, uses caveolae as one pathway for internalization in CHO cells co-transfected with caveolin cDNA and GH receptor cDNA [21]. The p85 regulatory subunit of PI_3-kinase was used as an additional marker of endocytotic compartments taking into account recent data showing that an early step in endocytosis of receptor-mediated albumin uptake was inhibited by overexpression of a truncated p85 subunit [22]. Fig. 5 indicates that whatever the stage of MEC polarization, the p85 ECL signal of highest intensity was clearly

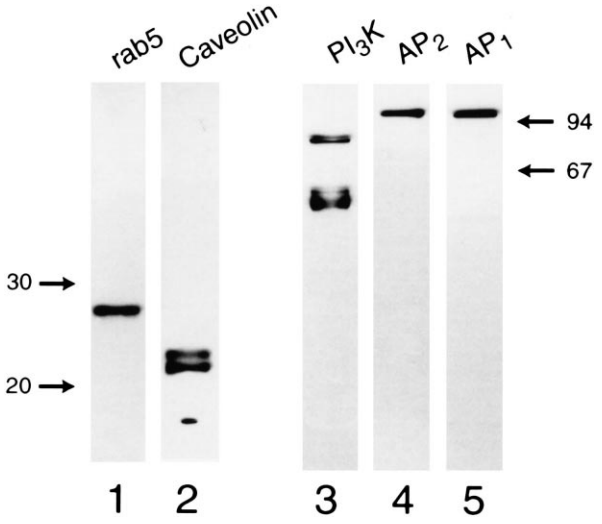

Fig. 4. Detection of the rab5 protein, caveolin, PI_3-kinase and the AP_2 and AP_1 adaptors in crude vesicles prepared from mammary tissue of lactating rabbits. Crude vesicles prepared as described in Section 2 were subjected to SDS–PAGE (lanes 1–2, 12% acrylamide, lanes 3–5, 10% acrylamide) and to Western blotting using antibodies directed against the rab5 protein, caveolin, PI_3-kinase and the AP_2 and AP_1 adaptors. Ten μg of proteins were loaded in each lane. The size of the molecular weight markers is indicated in kDa.

associated with fraction 3. Fig. 5 also shows that AP_2 and AP_1, markers of the endocytotic and exocytotic clathrin-coated vesicles, respectively, are concentrated in fractions of high density (interfaces 3 and 4). Accordingly, pictures obtained by electron microscopy after negative staining display 100 nm vesicles decorated with the clathrin coat characteristic of these cell compartments (data not shown). In non-polarized MEC, both markers are equally distributed in fractions 3 and 4. In contrast, gradient profiles obtained from polarized MEC indicate that fractions 3 and 4 are enriched with the AP_2 and AP_1 adaptors, respectively. The faint but significant signal detected with antibodies directed against AP_2 in low density fractions (interfaces 1 and 2) prepared from non-polarized cells is in agreement with previously published data showing that this protein can be used to identify non-coated vesicles as early endosomes [23]. It has to be noted that no signal was detected in fractions 1 and 2 of gradients performed with membrane extracts of polarized MEC.

Altogether these data bring evidence that in non-polarized MEC, at both 15°C and 37°C, prolactin is mainly transported by vesicles with biochemical characteristics of early endosomes. Upon membrane polarization, the intracellular transit of prolactin is clearly modulated by temperature. At 37°C, the hor-

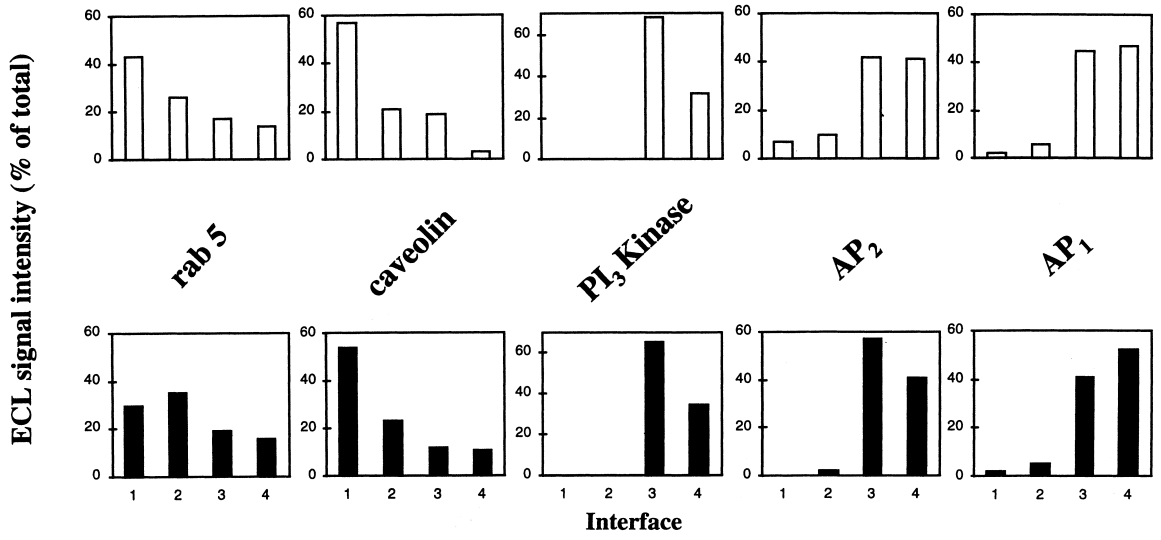

Fig. 5. Distribution of specific membrane markers in MEC organelles separated on sucrose density gradients. Crude vesicles prepared from mammary fragments of pregnant (upper) and lactating (lower) rabbits were loaded on a 5-step density gradient. After centrifugation at $35\,000 \times g$ for 4 h, fractions were collected at the four interfaces. The distribution of membrane markers was analyzed by Western blotting. Ten μg of proteins were loaded in each lane. Similar profiles were obtained in three independent experiments. Interface 1: $d = 1.119$ g/ml; interface 2: $d = 1.132$ g/ml; interface 3: $d = 1.152$ g/ml; interface 4: $d = 1.182$ g/ml.

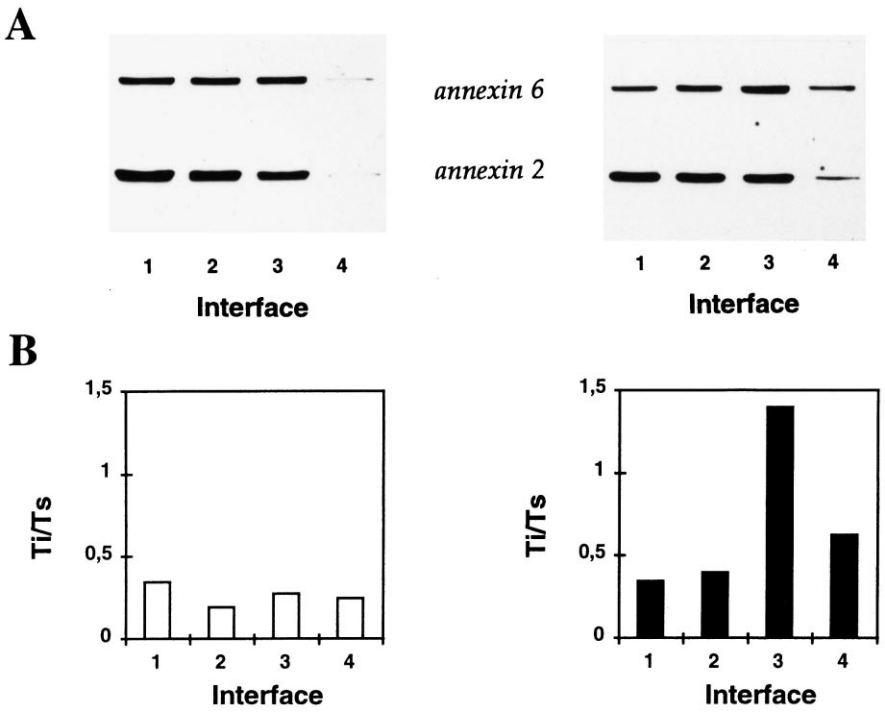

Fig. 6. A Recruitment of annexin VI and annexin II to MEC organelles. Each collected fraction of the 5-step gradients carried out with crude vesicles prepared from mammary tissue fragments was subjected to SDS–PAGE and Western blotting using antibodies directed against annexin VI and annexin II. Left and right profiles are obtained from pregnant and lactating rabbits, respectively. Ten μg of proteins were loaded in each lane. B: Distribution of annexin VI between TX100-insoluble and TX100-soluble subfractions obtained from MEC organelles. Each collected fraction of the 5-step gradients carried out with crude vesicles prepared from mammary fragments was subjected to TX100 treatment under mild conditions (1%, 4°C, 20 min). The left and right profiles were obtained from pregnant and lactating rabbits, respectively. Ordinate: ratios of the amounts of annexin VI recovered in the TX100-insoluble (Ti) and TX100-soluble (Ts) subfractions by quantification of ECL signals after Western blotting. Similar profiles were obtained in three independent experiments. Interface 1: $d=1.119$ g/ml; interface 2: $d=1.132$ g/ml; interface 3: $d=1.152$ g/ml; interface 4: $d=1.182$ g/ml.

mone is detected in cell compartments of high density (interface 4). At this interface, we collected clathrin-coated vesicles enriched with AP_1. This is in good agreement with previous electron microscopic investigations indicating that under these experimental conditions, the gold-labelled hormone and AP_1 are located in the apical region of the cell [1,24]. Such differences in MEC functioning upon establishment of membrane polarization matches well with data published by Gan et al. who showed that epithelial cell polarization modulates the transcytotic pathway of IgA [25]. Several years ago, Maurice et al. reported that in both primary liver cell cultures and hepatoma cell lines, the transport of canalicular and sinusoidal antigens was altered in the absence of the bile pole [26]. From studies on undifferentiated and differentiated HT29 colon carcinoma cells, it was shown that the intracellular trafficking of sphingolipids also changes upon cell polarization [27].

3.3. Annexin VI recruitment to MEC organelles

Annexins were detected in MEC more than two decades ago [28] but until now, most data concern their differential expression during mammary gland development [29,30] and malignancy [31,32]. To our knowledge, the only report concerning the subcellular distribution of annexins in membrane compartments of MEC is an immunocytochemical study where annexin II was detected at the apical pole of the cells, associated to vesicles that the authors thought to be either secretory vesicles or lipid droplets [33]. Having in hands distinct intracellular compartments, we initiated a biochemical study on the subcellular localization of annexin VI in non-polar-

ized and polarized MEC. Western blots displayed on Fig. 6A reveal that annexin VI is recruited to the membranes of all organelles isolated from non-polarized and polarized MEC but in both cases, the intensity of the ECL signals significantly drops in fraction 4. This probably reflects variations in lipid composition of the cell organelles collected at different gradient interfaces taking into account the membrane-binding characteristics of annexins. Preferential localization of annexin VI in the endocytotic compartments has already been reported. For example, annexin VI was detected in endothelial caveolae [34]. In hepatocytes, Jäckle et al. observed that annexin VI is an endosome marker [35] and very recently, Ortega et al. demonstrated by Western blotting and immunoelectron microscopy that, in rat liver, annexin VI is associated to endocytotic structures enriched with the rab5 protein [36]. Note the similar profile obtained with antibodies directed against annexin II (Fig. 6).

The ability of transport vesicles to form or pinch off from their membrane of origin into discrete vesicles via a process of budding and fission results from the segregation of membrane components into microdomains. In MDCK cells, these microdomains, insoluble in TX100 at 4°C, were considered to be sorting centers for proteins destined to be delivered to the apical plasma membrane [37]. As a logical starting point to specify the putative role of annexin VI in prolactin transport, its recruitment to microdomains of MEC compartments was studied. The different vesicles were solubilized with 1% TX100 at 4°C, and the TX100-insoluble (Ti) and TX100-soluble (Ts) subfractions were further analyzed by Western blotting (Fig. 6B). In membrane compartments prepared from mammary tissue of pregnant rabbits, the Ti/Ts ratio values ranged from 0.35 to 0.20 indicating that at this stage of cell polarization, annexin VI is poorly recruited to Ti membrane subdomains. Upon MEC polarization, the solvation characteristics of annexin VI by TX100 significantly changed. This event mainly concerns the gradient fraction enriched with endocytotic clathrin-coated vesicles (fraction 3), whose Ti/Ts ratio value reaches 1.4. It suggests a possible participation of annexin VI in the early steps of prolactin trafficking, in agreement with the results published several years ago by Lin et al. [38] who proposed a role for annexin VI in the budding events initiating intracellular traffic. It is also consistent with recent data on the requirement of annexin VI to disconnect the clathrin lattice from the spectrin membrane cytoskeleton during the final stages of budding in fibroblasts [39]. In the adrenocortical tissue, it was shown that annexin VI and dynamin, a GTPase involved in the pinching off process, are closely associated in endocytic coated vesicles [40]. On the contrary, Smythe et al. reported that endocytosis occurs independently of annexin VI in human A431 cells [41]. Our data support the former scenario.

Various studies have focused on annexin II and the annexin II/p11 complex as a potential mediator of Ca^{2+}-regulated secretion in adrenal chromaffin cells, but several lines of evidence indicate that annexin II is also a good candidate for an endocytotic mediator. Annexin II is associated with early endosomes of BHK cells [42] and a role for this protein in early endosome dynamics is supported by experiments using MDCK cells expressing an annexin II/p11 chimera [43]. As shown in Fig. 6A, annexin II was recovered in endocytotic organelles of both non-polarized and polarized MEC with, as observed for annexin VI, a very faint signal in the membrane fraction of highest density. In contrast, following TX100 treatment, annexin II is mostly recovered in Ts subfractions obtained from all types of membrane compartments, whatever the state of cell polarization (Ti/Ts < 0.5). These data support the hypothesis that different annexins most likely play different biological roles by acting on different membrane targets.

To our knowledge, this is the first report on the comparison of the transcytotic pathways between non-polarized and polarized MEC. The fact that upon MEC polarization, increased recruitment of annexin VI to membranes of endocytotic clathrin-coated vesicles correlates with changes in prolactin travelling supports the hypothesis that annexin VI may act as a sorting signal. Our data should contribute to a better understanding of the puzzling role of annexins in transcytosis.

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