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α_{S1}-casein is required for the efficient transport of β- and κ-casein from the endoplasmic reticulum to the Golgi apparatus of mammary epithelial cells

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SUMMARY

In lactating mammary epithelial cells, interaction between caseins is believed to occur after their transport out of the endoplasmic reticulum. We show here that, in α_{S1}-casein-deficient goats, the rate of transport of the other caseins to the Golgi apparatus is highly reduced whereas secretion of whey proteins is not significantly affected. This leads to accumulation of immature caseins in distended rough endoplasmic reticulum cisternae. Casein micelles, nevertheless, were still observed in secretory vesicles. In contrast, no accumulation was found in mammary epithelial cells which lack β-casein. In mammary epithelial cells secreting an intermediate amount of α_{S1}-casein, less casein accumulated in the rough endoplasmic reticulum, and the transport of α_{S1}-casein to the Golgi occurred with kinetics similar to that of control cells. In prolactin-treated mouse mammary epithelial HC11 cells, which do not express α_S-caseins, endoplasmic reticulum accumulation of β-casein was also observed. The amount of several endoplasmic reticulum-resident proteins increased in conjunction with casein accumulation. Finally, the permeabilization of rough endoplasmic reticulum vesicles allowed the recovery of the accumulated caseins in soluble form. We conclude that optimal export of the caseins out of the endoplasmic reticulum is dependent upon α_{S1}-casein. Our data suggest that α_{S1}-casein interacts with the other caseins in the rough endoplasmic reticulum and that the formation of this complex is required for their efficient export to the Golgi.

Key words: Mammary gland, Epithelial cell, Casein, Secretion, Endoplasmic reticulum

INTRODUCTION

During lactation, mammary epithelial cells (MEC) synthesise and secrete huge quantities of milk specific proteins and other milk components such as lipids and lactose. With regard to the protein content of milks, marked qualitative and quantitative (≈4 g/l to 200 g/l) differences have been observed between species. Except in primates, however, the main milk proteins are the caseins (Cns). The Cns are a family of four acidic phosphoproteins (α_{S1}-, α_{S2}-, β- and κ-Cn; for review see Swaisgood, 1992) the relative proportions of which also vary among species. Cns are found in milk as colloidal aggregates, the Cn micelles. Morphological data indicate that the aggregation of the Cns into Cn micelles is initiated in the trans-most cisternae of the Golgi apparatus. During the packaging of the Cns into secretory vesicles and transport to the apical plasma membrane, aggregation proceeds to give rise to the typical honeycombed mature Cn micelles. Aggregation of the Cns follows their phosphorylation on multiple phosphorylation sites in the Golgi apparatus and involves interaction of the phosphorylated residues with calcium phosphate. In line with this, the concentration of calcium and phosphate in milk is highly correlated with the Cn content of milk. Therefore, one biological function of the Cn micelle might be to supply the young with calcium and phosphate. On the other hand, it has been proposed that the binding of calcium phosphate to the Cns could protect the mammary gland from pathological calcification (Holt, 1992).

The specific arrangement of the Cns in the micelle has not yet been established. Hence, several models of the Cn micelle structure have been proposed (for review see Rollema, 1992). In some models, the micelle is described as an inhomogeneous aggregate with the 'calcium-sensitive' caseins (α_{S1}-, α_{S2}- and β-Cn) preferentially located within the core of the micelle, the outer layer consisting predominantly of κ-Cn (coat-core models). An alternative view is that Cn micelles form as a result of the aggregation of subunits, the so-called Cn submicelles, and spherical particles, tentatively identified as those premicellar subunits, can be observed as early as the cis-Golgi (Clermont et al., 1993). However, the detailed mechanisms of the formation of the putative submicelles, as well as their structure, remain to be elucidated. Moreover, much is still to be learned about the role of the individual Cn in the aggregation process. Finally, the possible role of Cns aggregation in their transport from the ER to the Golgi apparatus and/or from the trans-Golgi network (TGN) to the secretory vesicles has not directly been investigated.

Disruption of a gene by homologous recombination is a powerful approach to address the function of a protein but separate disruption of each of the Cn encoding genes might be necessary in order to elucidate the mechanisms of Cn micelle formation and Cns secretion. An alternative, or at least an additional approach to elucidate the role of the individual Cns in this process is to study the secretion of milk proteins in well-characterised animals naturally deficient in a specific Cn. For obvious economical reasons, the genetic polymorphism of milk proteins has been extensively studied in cattle breeds and null phenotypes for β-Cn (Mahé and Grosclaude, 1993) and α_{S1}-Cn (Grosclaude et al., 1987) have been found in the goat. In fact, the goat *α_{S1}-Cn* gene presents an extensive polymorphism controlled by at least seven alleles (for review see Martin and Grosclaude, 1993). This polymorphism is associated to quantitative variations of α_{S1}-Cn in milk (Grosclaude et al., 1987). Alleles *α_{S1}-CnA*, *B* and *C* are associated with a high α_{S1}-Cn content in milk (3.6 g/l per allele), allele *α_{S1}-CnE* with an intermediate level (1.6 g/l per allele), and alleles *α_{S1}-CnG* and *F* with a low content (0.6 g/l per allele). Finally, allele *α_{S1}-CnO* is a true null allele. α_{S1}-CnA, B, C and E variants only differ in amino acid substitutions (Brignon et al., 1989, 1990). Concerning the reduced amount of α_{S1}-Cn in the milk of the α_{S1}-CnE variant, a reduction of the transcripts has been observed (Jansa Pérez et al., 1994), possibly due to a decrease of the mRNA stability. Elucidation of the primary structures of mutants F and G revealed internal deletions in the protein of 37 and 13 amino acids, respectively (Brignon et al., 1990; Martin and Leroux, 1994). These deletions are due to the skipping of exon 4 in α_{S1}-CnG (Martin and Leroux, 1994) and of exons 9 to 11 in α_{S1}-CnF, during the processing of the primary transcripts (Leroux et al., 1992). In the later case, it has been proposed that this exon-skipping might be responsible for the observed decrease of the corresponding mRNA and hence for the reduction of α_{S1}-Cn in milk. Moreover, the deletion includes the multiple phosphorylation site of the molecule. This will obviously result in a dramatic modification of the physico-chemical properties of the proteins.

We took advantage of this extensive polymorphism at the *α_{S1}-Cn* locus in goat to obtain information on the potential role of α_{S1}-Cn in the transport of the other Cns in the secretory pathway of MEC, and to address its function in Cn micelle formation and/or secretion. To achieve this, the transport and secretion of the Cns were analysed at the morphological and biochemical level in MEC from homozygous animals producing high (*α_{S1}-CnA/A* goat), intermediate (*α_{S1}-CnE/E* goat) or low amounts of α_{S1}-Cn in milk (*α_{S1}-CnF/F* goat), as well as in goats which do not express this Cn (*α_{S1}-CnO/O* goat). Results have been compared to those obtained with goats which do not express β-Cn (*β-Cn0/0* goat).

MATERIALS AND METHODS

Reagents

RPMI 1640 medium, RPMI 1640 for metabolic labelling, bovine insulin, murine EGF and antibiotics were obtained from Life Technologies (Cergy, France). FCS was from Biosys S.A. (Compiègne, France). Ovine prolactin (PRL, NIADKK o-PRL-17, 31 i.u./mg) was kindly provided by A. F. Parlow (NIDDK's National Hormone and Pituitary Programme, Bethesda, MD, USA). Unless otherwise indicated, chemicals were from Life Technologies and Sigma Aldrich (St Quentin Fallavier, France).

Animals, cells and antibodies

French-Alpine or Saanen goats, homozygous at the *α_{S1}-Cn* locus were obtained from the Station Caprine de Moissac (Ste Croix-Vallée, France). The Pyrenean homozygous *β-Cn* null goat was from the Conservatoire du Patrimoine Biologique Régional de Midi Pyrénées, Station d'Amélioration Génétique des Animaux, Institut National de la Recherche Agronomique (INRA, Toulouse, France). Genotypes were determined using a PCR-based allele-specific typing procedure (Leroux et al., 1993; Martin and Leroux, 1994). The mouse mammary epithelial cell line HC11 (Ball et al., 1998) was obtained from Dr B. Groner (Freiburg im Breisgau, Germany). Polyclonal antibodies against goat α-lactalbumin (used at a dilution of 1:1000), β-lactoglobulin (either used at a dilution of 1:2500 for immunoblotting or of 1:300 for immunofluorescence) and Cn (used at a dilution of 1:200) were obtained from M.-F. Mahé (Jouy-en-Josas, France). Immunoblotting showed that this later antibody, raised against α_{S2}-Cn, cross-reacts with other Cns, notably with β- and κ-Cn. The antiserum against goat κ-Cn, obtained from Dr C. Printz (Jouy-en-Josas, France), was either used at a 1:2000 dilution for immunoblotting or at a 1:200 dilution for immunoelectron microscopy. The antibody against mouse Cn (#7781) was a gift from Prof. M. C. Neville (Denver, CO, USA), and was used at a dilution of 1:200. Antibodies against mouse milk proteins (RAM/MSP, Nordic Immunological laboratories, Tilburg, The Netherlands) was used at a dilution of 1:5000. The antibody against the protein disulphide isomerase (PDI) carboxy terminal tail KAVKKDEL (KAVK, Vaux et al., 1990) was kindly provided by Dr S. Fuller (Heidelberg, Germany), and used at a 1:2000 dilution. Antibodies against canine calnexin and human calreticulin were from StressGen Biotechnologies Corp. (Victoria, BC, Canada) and used at a 1:1000 dilution. All antibodies were from rabbit. Secondary antibodies were HRP-conjugated (used at a 1:5000 dilution) or rhodamine-conjugated goat anti-rabbit IgG (used at a 1:200 dilution), both from Jackson Immunoresearch Lab., Inc. (Avondale, PA, USA), and FITC-conjugated goat anti-rabbit IgG (used at a 1:300 dilution) from Sanofi Diagnostics-Pasteur (Marnes-la-Coquette, France) or gold-conjugated goat anti-rabbit IgG (used at a 1:200 dilution) from Aurion (Wageningen, The Netherlands).

Preparation of mammary gland fragments

Samples of mammary gland from lactating goats homozygous for distinct *α_{S1}-Cn* alleles were removed from the animal immediately after sacrifice, cut into 1-2 mm width slices and finely minced using a home-made multi-mounted razor blade device or cut manually into ≈1 mm^3 fragments with a razor blade. In the case of *β-Cn0/0* goat, the animal was anaesthetized, the skin of the mammary gland was incised and small fragments of tissue were taken using a biopsy needle (18G gauge, TruCut Biopsy Needle, Baxter Healthcare Corp., Deerfield, Ill, USA).

Metabolic labelling and analysis of the maturation or the secretion of the Cns

All incubations were in medium supplemented with 5 g/l Na-acetate, at 37°C in 95% O_2/5% CO_2. In standard procedure, 50 mg of explants were used per experimental condition. Mammary fragments were preincubated for two periods of 15 minutes in methionine/cysteine-free RPMI 1640 and then pulse labelled for 5 minutes with fresh methionine/cysteine-free RPMI 1640 containing 1.85 MBq/ml (50 μCi/ml) L-[^{35}S]methionine from Pro-mix™ (Amersham Pharmacia Biotech, Les Ulis, France). In some experiments, fragments were preincubated and pulse labelled with 0.74 MBq/ml (20 μCi/ml) L-[4, 5-^{3}H]leucine (ICN, Orsay, France), both in leucine-free RPMI 1640 medium. In pulse-chase experiments, explants were pooled during the preincubation and pulse labelling periods.

At the end of the pulse, explants were quickly washed in regular

RPMI 1640 containing twice the normal concentration of methionine and cysteine or leucine, and divided into 50 mg (pulse) or 100 mg pools (pulse and chase). To chase the label, explants were further incubated for 60 or 120 minutes in the above medium. Chase medium was collected, centrifuged for 5 minutes at 240 ***g*** or filtered though a 5 μm syringe filter (Minisart NML, Sartorius S.A., Palaiseau, France) to remove cellular debris. Radioactive proteins in chase medium or associated with half of the corresponding explants were quantitated as previously described (Clegg et al., 1998) after precipitation at pH 4.6 or TCA precipitation (10% final concentration), respectively. Remaining tissue or tissue collected at the end of the pulse were resuspended in ice-cold TBS/PMSF (25 mM Tris-HCl, pH 7.4, 4.5 mM KCl, 137 mM NaCl, 0.7 mM Na_2HPO_4, 0.5 mM PMSF) and pelleted by a brief centrifugation at 700 ***g*** and 4°C, three times. All subsequent steps were performed at 0-4°C. Tissue was incubated for 30 minutes in 1 ml of 12 mM Tris-HCl, pH 7.8, 0.4% (w/v) SDS, and cells were further lysed by sonication. The resulting cell lysate was centrifuged for 15 minutes at 15,000 ***g*** and proteins precipitated from supernatant were analysed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and fluorography.

Analysis of the RER content

Pieces of mammary gland, snap frozen in liquid N_2 after sacrifice and stored at −20°C, were broken into small fragments (≈0.5 cm^3), further frozen and crushed to a fine powder, both under liquid N_2. All subsequent steps were performed at 4°C. Tissue powder was resuspended in 10 vols 0.25 M sucrose containing 10 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid (Hepes)-KOH, pH 7.2, 1 mM EDTA, 0.5 mM PMSF, plus a protease inhibitor cocktail (Sigma Aldrich, P8340) at a 1:500 dilution, and cells were homogenized using a glass homogenizer (DUALL® 23, Kontes Glass Co, NJ, USA). The homogenate was filtered through a piece of a 150 μm pore polypropylene mesh (ZBF, Rüschlikon, Switzerland) and centrifuged for 10 minutes at 1000 ***g***. Aliquot of the resulting supernatant, referred to as post-nuclear supernatant (PNS), was assayed for protein concentration using the Peterson's method and identical amount of protein from each PNS was analysed by SDS-PAGE or 2D-PAGE followed by either protein staining or immunoblotting.

For identification of proteins accumulated in the RER, the proteins contained in the PNS prepared from MEC of α_{S1}*-CnO/O* goat were separated by 2D-PAGE and transferred to ProBlott™ membrane (Applied Biosystems, CA, USA) in 3-[cyclohexylamino]-1-propanesulfonic acid buffer according to the manufacturer's recommendations. The membrane was stained with Coomassie Blue, destained, extensively washed in H_2O and spots of interest were excised from the membrane. N-terminal amino acid sequence was obtained with a 494A Procise Edman sequencer (Applied Biosystems).

The solubility of the Cns accumulated in RER vesicles was analysed after permeabilization of intracellular membranes with saponin (Chanat and Huttner, 1991). Explants prepared from the mammary gland of α_{S1}*-CnA/A*, α_{S1}*-CnE/E*, or α_{S1}*-CnF/F* goat were pulse labelled for 5 minutes with [^{35}S]Pro-mix™, chilled on ice, and washed three times with ice-cold TBS/PMSF as described above. All subsequent steps were performed at 4°C. Explants were further washed once in homogenization buffer (HB: 0.25 M sucrose, 1 mM EDTA, 1 mM Mg acetate, 10 mM Hepes-KOH, pH 6.8, 0.5 mM PMSF) and homogenized in 1 ml of HB with 8 strokes of a tissue grinder (AA2 Teflon/glass, Thomas Scientific™, NJ, USA) coupled to a motor drive (Heidolph-Elektro GmbH, Kelheim, Germany) set to ≈300 rpm. A PNS was prepared from the homogenate as described above, divided into 200 μl aliquots, and [^{35}S]methionine/cysteine-labelled vesicles were pelleted by centrifugation at 200,000 ***g*** for 30 minutes. Pellets were resuspended in 100 μl of 0.25 M sucrose containing 10 mM MES-NaOH, pH 7.4, 30 mM KCl and a protease inhibitor cocktail (see above), in the absence or presence of 1 mg/ml saponin plus 100 mM KCl, supplemented or not with either 1 mM EDTA or 1 mM EDTA plus 10 mM MgATP. Samples were incubated for 30 minutes, centrifuged as above, and pellets and supernatants were analysed by SDS-PAGE followed by fluorography.

De-phosphorylation of Cn from milk

Cn fraction was prepared from the milk of individual α_{S1}*-CnA/A* or α_{S1}*-CnE/E* goats, collected at one milking. Frozen milk was thawed and kept at room temperature for 1.5 hours. An aliquot was taken from below the fat, diluted 3 times with 10 mM Tris-HCl, pH 7.4, supplemented with 10 mM $CaCl_2$, and centrifuged for 15 minutes at 15,000 ***g*** at room temperature. The pellet, which contained the vast majority of the Cns, was dissolved in 1 ml of phosphatase assay buffer (40 mM piperazine-*N*, *N*′-bis-[2-ethanesulfonic acid], pH 6.0, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM PMSF, protease inhibitor cocktail as above). After sedimentation of insoluble particles, supernatants were further diluted to a final protein concentration of ≈0.5 mg/ml and aliquots (0.5 ml) were supplemented with either 1 μg potato acid phosphatase (EC 3.1.3.2, Sigma Aldrich; stored at −70°C, at 1 mg/ml in phosphatase storage buffer: 10 mM Hepes, pH 7.4, 0.5 mM $MnCl_2$, 0.5 mM dithiothreitol, 50% w/v glycerol) per 50 μg of protein or equivalent volume of phosphatase storage buffer (control). After a 5 hours incubation at 30°C, proteins were subjected to TCA precipitation and resuspended in 2D-PAGE lysis buffer. In addition, aliquots of acid phosphatase-treated proteins and of the PNS prepared from the mammary gland of an α_{S1}*-CnO/O* goat were mixed. Samples were analysed by 2D-PAGE followed by protein staining. 2D-PAGE analysis of phosphatase-treated [^{32}P]orthophosphate-labelled Cns demonstrated that the above treatment leads to the production of phosphate-free Cns which, at least in the case of α_{S1}- and β-Cn, co-migrate with their corresponding ER forms (data not shown). The N-terminal sequences of de-phosphorylated α_{S1}-CnA/A and α_{S1}-CnE/E was determined as described above.

Analysis of the intracellular transport of Cn in HC11 cells

Culture of HC11 cells and induction of Cn synthesis by lactogenic hormones were essentially as described by Ball et al. (1998). HC11 cells were maintained in RPMI 1640/10% FCS supplemented with 4 μg/ml insulin, 10 ng/ml EGF, 100 i.u./ml penicillin and 100 μg/ml streptomycin, at 37°C in 5% CO_2 in air. For the analysis of Cns secretion, HC11 cells were seeded on 24.5 mm diameter filters (0.4 μm pore size, Transwell®-Clear, Costar, Cambridge, MA, USA) at about the density achieved by confluent cells on plastic, and grown in the above medium for 2 days. Cells were further cultured for 2 days in RPMI 1640/10% horse serum supplemented with insulin, 10^{-6} M hydrocortisone and antibiotics, and for 5-7 days in the same medium in the absence (control) or presence of 5 μg/ml PRL to induce Cn synthesis. Growth factors and hormones were only added to the basal side of the cells.

To analyse Cn expression and secretion, cells were washed three times with prewarmed RPMI 1640, and incubated for 1 or 3 hours in serum-free RPMI 1640 supplemented with insulin, hydrocortisone, antibiotics, in the absence or presence of PRL. Apical and basal media were collected, centrifuged for 5 minutes at 800 ***g*** and 4°C, and supernatants were TCA precipitated. Cells were washed 3 times using ice-cold RPMI 1640 and lysed in situ in Laemmli sample buffer (Laemmli, 1970). Media and 1:10th of the cell extract were analysed by SDS-PAGE followed by immunoblotting.

EM, immunogold EM and immunofluorescence

Tissue fragments were fixed and processed for EM, immunogold EM or immunofluorescence as previously described (Pauloin et al., 1997; Clegg et al., 1998). For EM analysis of filter-grown HC11 cells, cells were briefly washed with 0.1 M sodium cacodylate buffer, pH 7.2, and fixed for 1 hour with 2% glutaraldehyde in the same buffer. Filters of the cell culture inserts were cut and processed as tissue fragments. Immunofluorescence analysis of HC11 cells was essentially as described by Fiedler at al. (1995).

Gel electrophoresis, immunoblotting and quantitation of radioactivity in protein

SDS-PAGE was performed according to the method of Laemmli (1970), using 13% gels. For the analysis of proteins from goat milks, an aliquot was taken from below the milk fat and the equivalent of 0.4 μl of milk per lane was loaded. As a positive control for immunoblotting of mouse milk proteins, 10 μl of a 1:5000 dilution of crude mouse milk was loaded. 2D-PAGE was essentially performed according to the method of O'Farrell (1975), using a mixture of Ampholines (Amersham), pH 3.5-9.5 (2.0%, v/v), pH 3.5-5.0 (2.5%, v/v) and pH 5.0-7.0 (2.5%, v/v) in the first dimension. Experimental isoelectric points were measured on a sample-free IEF gel after incubation of 1 cm width gel pieces in H_2O. For the second dimension 13% gels were used. Gels were stained, destained and processed for fluorography as previously described (Lee and Huttner, 1983). When appropriate, [^{35}S]methionine/cysteine-labelled proteins were quantified by densitometric scanning (ImageMaster™, Pharmacia) of the fluorograms. Immunoblotting was as described by Rosa et al. (1989), except that the blocking of the nitrocellulose (BA 83, Schleicher & Schuell GmbH, Dassel, Germany) was by incubation for 1 minute in polyvinyl alcohol (1 μg/ml) in PBS (Miranda et al., 1993) followed by one-hour incubation in PBS containing 10% low fat milk powder. Incubations with antibodies were in PBS/milk supplemented with 0.3% Tween-20, and immunoreactive proteins were revealed by incubation with HRP-conjugated goat anti-rabbit IgG antibodies followed by enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham).

RESULTS

The RER of MEC from αS1-Cn-deficient goats is distended

EM analysis of the tissues obtained from *αS1-CnA/A* goat (Fig. 1A) exhibited the characteristic morphological features of lactating MEC (Clermont et al., 1993; Pitelka and Hamamoto, 1983); an expanded RER, often seen in parallel lamellae, a well-developed Golgi apparatus with distended trans-cisternae and multiple vesicular structures, and abundant swollen secretory vesicles containing linear electron dense filaments, tentatively identified as aggregated Cn submicelles, and Cn micelles, both suspended in an electron-lucent fluid. As Fig. 1C shows, the RER of MEC from *αS1-CnO/O* goat, which does not express αS1-Cn, was dramatically distended, its lumen being filled by a granular electron dense material. The morphology of MEC from *αS1-CnF/F* goat, a low-producing variant, was virtually identical (data not shown). Finally, the RER of MEC from *αS1-CnE/E* goat, which produce intermediate level of αS1-Cn in milk, was only moderately distended (Fig. 1B). Interestingly, in tissue obtained from *β-Cn0/0* goat, the RER was slightly more fragmented but not distended (Fig. 1D). Hence, the remarkable dilation of the RER observed in αS1-Cn-deficient MEC, most likely due to accumulation of proteins in this compartment, did not simply reflect the lack of one of the Cn but was specific for αS1-Cn. Moreover, accumulation of protein in the RER was inversely correlated to the level of production of αS1-Cn in milk. These results suggested a role for αS1-Cn in milk protein secretion.

One can hypothesize that modification of the Cn content would perturb Cn micelle formation. Nevertheless, Cn micelles (arrowheads) were observed in secretory vesicles and in the lumen of the acini of all types of αS1-Cn-deficient goats (Fig. 1A-C; *αS1-CnF/F*, data not shown), and of *β-Cn0/0* goat (Fig. 1D). This finding demonstrates that the formation of Cn micelles is neither dependent upon the presence of αS1-Cn nor of β-Cn, as was previously shown in β-Cn-deficient mice (Kumar et al., 1994).

Identification of the proteins which accumulate in the RER of MEC from αS1-Cn-deficient goats

In order to identify the proteins that accumulate in the RER of αS1-Cn-deficient MEC we first investigated the localisation of milk proteins in MEC from goat which either produce high or low amounts of αS1-Cn in milk. Immunofluorescence showed that the Cns were essentially located in the supra nuclear part of MEC from *αS1-CnA/A* goat (Fig. 2A), i.e. in the region of the cell containing the Golgi apparatus and secretory vesicles. A general increase in Cn immunoreactivity was observed in MEC from *αS1-CnF/F* goats (Fig. 2B). Moreover the immunolabelling, consistent with the dilation of the RER in this variant, was found in the whole cytoplasm. In contrast, the signal for β-lactoglobulin (Fig. 2C and D) and α-lactalbumin (data not shown) was essentially similar in MEC from *αS1-CnA/A* and *αS1-CnF/F* goats. The analysis of MEC from *αS1-CnO/O* goat for both Cns and β-lactoglobulin (data not shown) gave essentially the same results as those described above for *αS1-CnF/F* goat. These comparisons indicated that accumulation of secretory proteins in the ER of goats bearing alleles associated with a reduced αS1-Cn content in milk affected the Cns but not the main whey proteins.

To analyse further the proteins which accumulated in the ER of αS1-Cn-deficient goats, proteins contained in PNS prepared from mammary tissue obtained from various goat variants and in the milks of the corresponding animals were analysed by SDS-PAGE followed by Coomassie Blue staining. The decrease of the band with an M_r of ≈32000 in the milk from *β-Cn0/0* goat (Fig. 3, Milk, β0) clearly examplified the lack of β-Cn. The M_r 32000 band which is observed in this sample most likely corresponded to αS1-Cn which, as shown below (see Fig. 6, Control and Fig. 7A, Chase), can only be separated from β-Cn by two-dimensional electrophoresis (2D-PAGE). For this reason and because β-Cn is the major Cn in goat milk, the decrease or lack of αS1-Cn in the milk of αS1-Cn-deficient goats were, in contrast, not clearly viewed. As to the tissues, two proteins with M_r of ≈27000 and 30000 were found in huge quantities in the PNS prepared from *αS1-CnF/F* and *αS1-CnO/O* mammary gland (Fig. 3, PNS, F and O, arrows), the protein pattern of these two PNS being very similar. These two proteins were also observed in substantial amount in the PNS prepared from the *αS1-CnE/E* goat but their relative proportion was much less, as compared to the two preceding genotypes (Fig. 3, PNS, E). It is noteworthy that none of these molecular forms had its equivalent in milks. These proteins were also not detected in the PNS prepared from *β-Cn0/0* goat tissue (Fig. 3, PNS, β0).

The fact that the relative amount of the proteins with M_r of 27000 and 30000 correlated with the extend of dilation of the RER observed in the MEC from αS1-Cn-deficient animals strongly suggested that they corresponded to those proteins filling the distended RER saccules. To identify these proteins, proteins of a PNS prepared from the mammary gland of *αS1-CnO/O* goat were separated by 2D-PAGE and relevant proteins were either subjected to N-terminal sequencing or identified by immunoblotting. Fig. 4A shows that the two major accumulated proteins had an isoelectric point of ≈4.8 and 4.6,

respectively. After transfer to ProBlott™, the numbered spots were excised from the membrane and an identical N-terminal amino acid sequence was obtained from spot number 2 and 3 (Table 1). A BLAST search (Altschul et al., 1990) of the NCBI database revealed that the recovered sequence had 100% identity only with the N-terminal sequence of goat and sheep β-Cn. No sequence information was obtained from peptide number 4. This protein, however, was identified by immunoblotting as κ-Cn (Fig. 4B, κ-Cn). Finally, α-lactalbumin and β-lactoglobulin were also identified by immunoblotting (Fig. 4B, LA and LG). This permitted to confirm that, in contrast to the Cns, the amount of these milk whey proteins was not significantly increased in MEC from α_{S1}-Cn-deficient goats (Fig. 4A, compare LA and LG in A/A and O/O). These results indicated that, among secretory proteins synthesised by MEC, only members of the Cn family of protein, namely β-Cn and κ-Cn, accumulated significantly in MEC from α_{S1}-Cn-deficient animals. Finally, two lines of

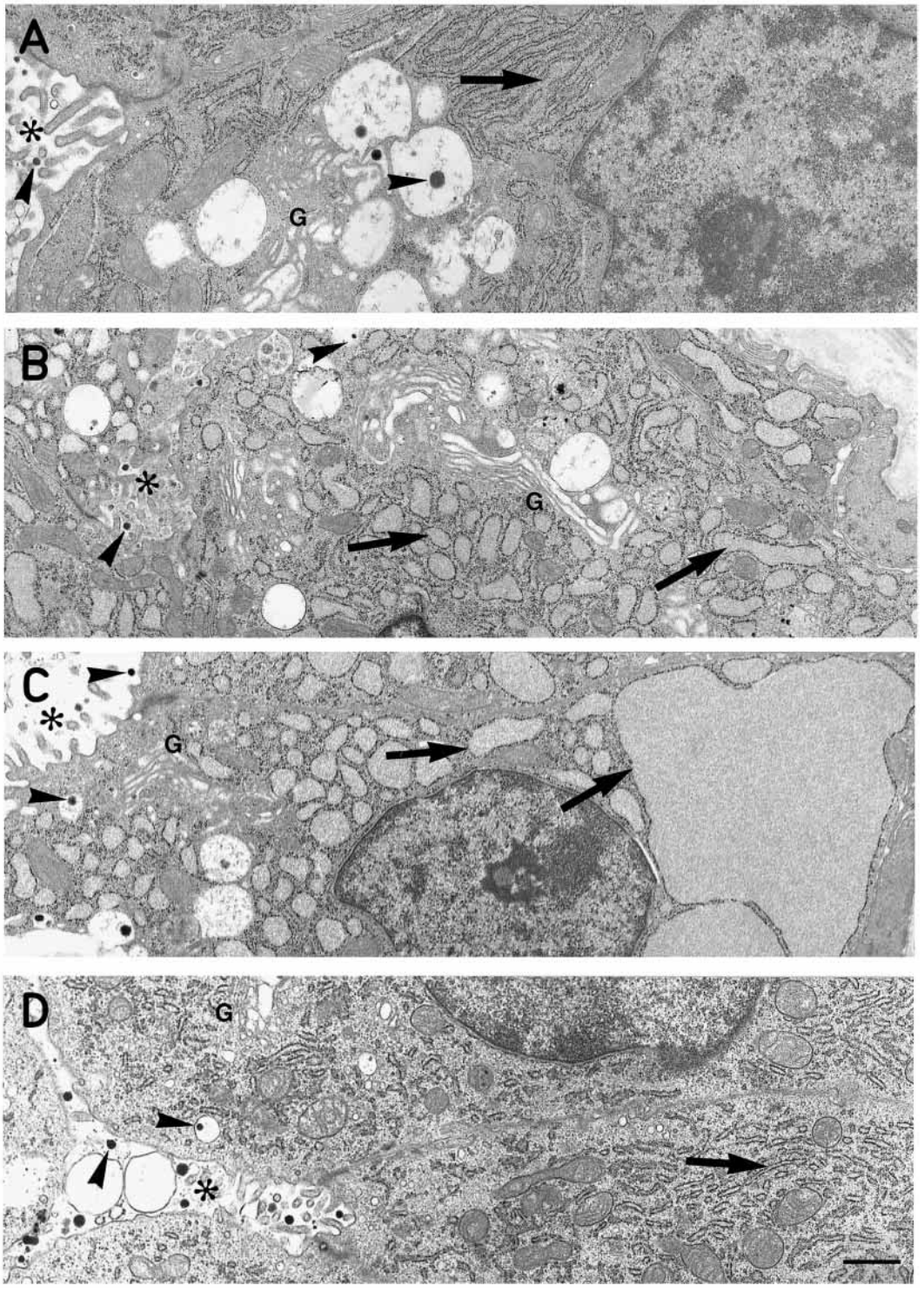

Fig. 1. Morphology of MEC from lactating goats homozygous for distinct alleles at the *α_{S1}-Cn* or *β-Cn* locus. Fragments of the mammary gland of lactating goat homozygous for allele *A* (A), *E* (B) or *O* (C) at the α_{S1}-Cn locus, or for allele *β0* at the *β-Cn* locus (D), were fixed and processed for EM. Note the remarkable enlargement of the cisternae of the RER (arrows) of MEC from *α_{S1}-CnO/O* goat (C). In *α_{S1}-CnE/E* goat (B), RER cisternae are also distended but to a lesser extent. In both α_{S1}-Cn-deficient MEC, a granular electron-dense material fills the lumen of the RER. Cn micelles (arrowheads) are present in the secretory vesicles and in the lumen (asterisk) of the acini of each animal. G: Golgi. Bar, 1 μm.

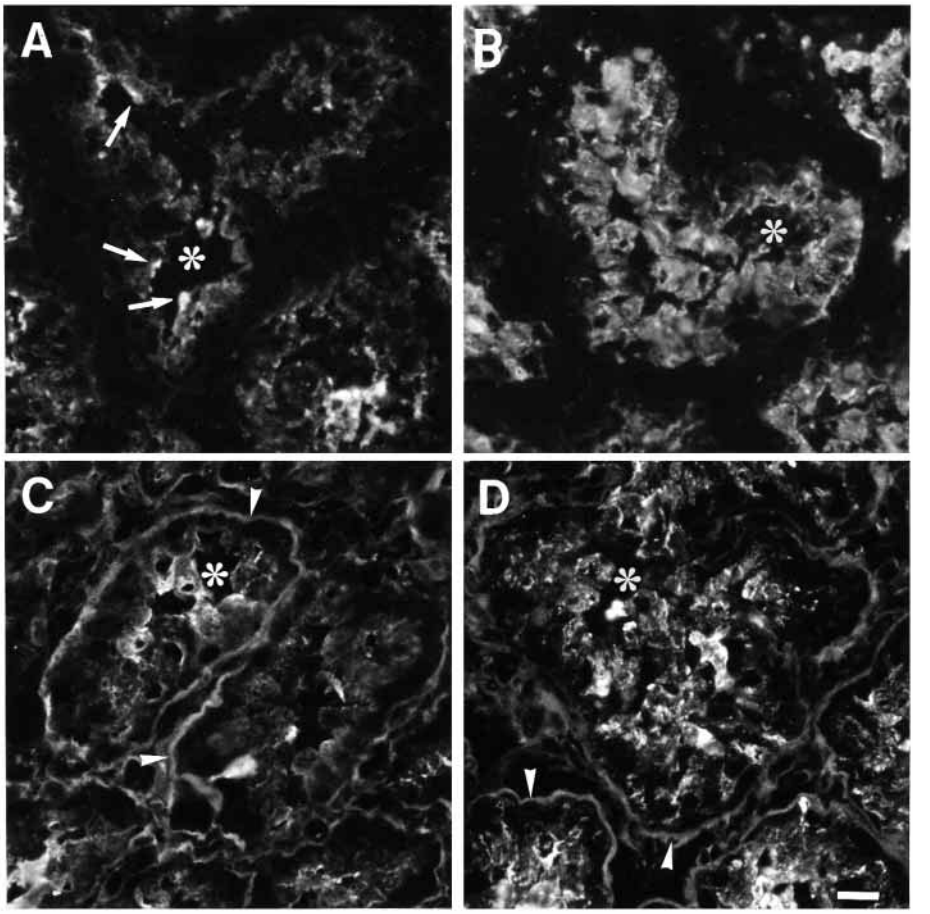

Fig. 2. Immunofluorescence localisation of lactoproteins in mammary acini of goat homozygous for distinct α_{S1}-*Cn* alleles. Fragments of the mammary gland of α_{S1}-*CnA/A* (A and C) or α_{S1}-*CnF/F* goats (B and D) were fixed and immunolabelled with either a rabbit anti-goat Cn antiserum (A and B) or a rabbit anti-goat β-lactoglobulin antiserum (C and D) and FITC-conjugated goat anti-rabbit IgG. In α_{S1}-*CnA/A* goat, Cn (arrow) are located at the cellular apex of MEC whereas they are detected in the whole cytoplasm in MEC from α_{S1}-*CnF/F* goat. In contrast the distribution of β-lactoglobulin is similar in both goat variants. Arrowhead: basal membrane. Asterisk: lumen. Bar, 10 μm.

evidence showed that these proteins essentially accumulated in the ER. First, using an antibody against goat κ-Cn we confirmed by immunogold labelling that this protein was exclusively located inside the compartments of the secretory pathway of α_{S1}-*CnO/O* MEC, gold particles being notably observed over the whole electron dense material within the distended RER cisternae (Fig. 5). Second, 2D-PAGE analysis of the proteins prepared from [^{32}P]orthophosphate-labelled explants from α_{S1}-Cn-deficient goats confirmed that the molecular forms of the Cns which accumulated are not phosphorylated, i.e. have not reached the Golgi apparatus (data not shown). Our observation that the electrophoretic mobilities of the accumulated Cns was modified as compared to milk was consistent with this. Taken together, these data suggested that the absence or deficiency of α_{S1}-Cn selectively altered the transport of the Cns, possibly at the exit of the ER, causing dilation of the RER so as to accommodate the increased amount of these proteins.

The amount of a few other proteins (M_r of ≈94000, 78000 and 55000) increased slightly but specifically in the PNS prepared from MEC which were shown to contained distended RER saccules (see Fig. 3, dots). Both for this reason and because of their molecular mass, we suspected that they

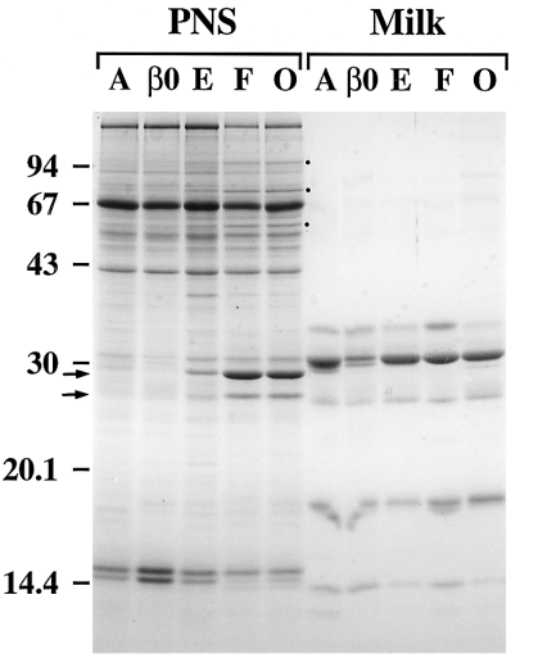

Fig. 3. Two major proteins accumulate in MEC from α_{S1}-Cn-deficient goats. A PNS was prepared from the mammary gland of either α_{S1}-*CnA/A* (A), *β-Cn0/0* (β0), α_{S1}-*CnE/E* (E), α_{S1}-*CnF/F* (F), or α_{S1}-*CnO/O* (O) goats. An aliquot of each PNS (PNS) and of the milk (Milk) obtained from goat bearing the same alleles were analysed by SDS-PAGE followed by Coomassie Blue staining. Note that none of the major proteins which accumulate in tissues of α_{S1}-Cn-deficient animals (arrows) are present in milk. Dots indicate minor proteins which increase in amount in α_{S1}-Cn-deficient goats and which were identified as ER-resident proteins. Positions of the molecular mass markers (kDa) are indicated on the left.

Table 1. Proteins identified by N-terminal sequencing

Spot No.	N-terminal sequence	Identity
1	EEEDKKEDVGTVVGIDLGT	BiP
2	REQEELN	β-Cn
3	REQEELN	β-Cn
4	Blocked	κ-Cn*

Proteins contained in a PNS prepared from the mammary gland of an *α_{S1}-CnO/O* goat were separated by 2-D PAGE, transferred onto ProBlott™ membrane, stained, and numbered proteins (see Fig. 4A, right) were subjected to N-terminal sequencing. *Protein no. 4 was identified by immunoblotting (see Fig. 4B).

were ER-resident proteins. In confirmation, the N-terminal sequence obtained from the polypeptide 1 (Fig. 4A and Table 1) was identical to that of mammalian immunoglobulin heavy chain binding protein (BiP; Ting and Lee, 1988). The proteins designated PDI and CRT (Fig. 4A, O/O), which also increased in MEC lacking α_{S1}-Cn, were respectively identified by immunoblotting as protein disulphide isomerase (Fig. 4B, PDI) and calreticulin (data not shown). In contrast, calnexin, which was also detected by immunoblotting, was not visible on these Coomassie blue stained gels (Fig. 4A, O/O, circle CNX).

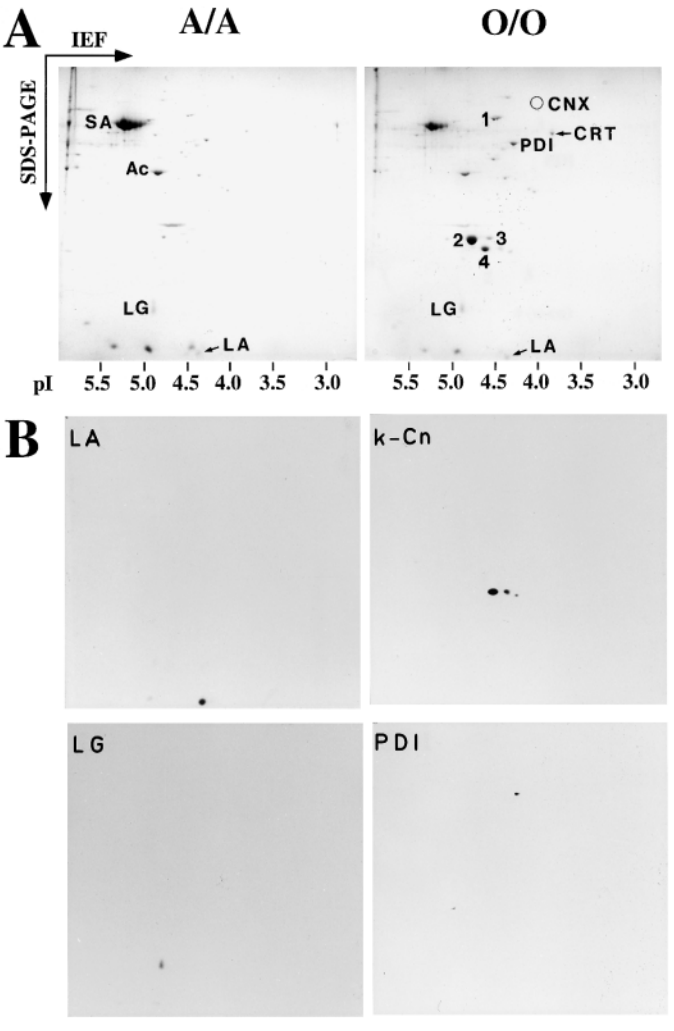

Table 2. Secretion of Cn is slowed down in α_{S1}-Cn deficient goats

Goat	% Cn secreted into medium	
	60 minutes chase	120 minutes chase
A/A (*n*=3)	19.8±2.9	27.5±4.7
E/E (*n*=2)	5.0±2.5	20.8±2.3
F/F (*n*=2)	6.6±1.0	12.4±0.9
O/O (*n*=2)	8.7±1.6	12.3±1.4

Mammary gland explants prepared from goats of the indicated *α_{S1}-Cn* genotypes were pulse labelled either for 5 minutes with [^{35}S]Pro-mix™ or for 3 minutes with [^{3}H]leucine, and chased for 60 or 120 minutes. At each chase time point, tissues and chase media were collected. Radioactive Cns from media and total proteins associated with the tissues were quantified. The [^{35}S]methionine/cysteine- or [^{3}H]leucine-labelled Cns in the medium was expressed as a percentage of the total (sum of cell-associated proteins plus medium Cns). For each condition, the mean is given. The number *n* of animals is indicated in parentheses. Either the variation of the single values from the mean (*n*=2) or the s.d. (*n*=3) is shown.

α_{S1}-Cn deficiency affects the secretion of the Cns

Having obtained several lines of evidence that α_{S1}-Cn deficiency causes accumulation of β-Cn and κ-Cn in the ER, it was tempting to speculate that this phenomenon would be associated to a reduction of the secretion of the Cns. Explants were pulse-labelled, chased for either 60 or 120 minutes, and radioactive Cns (Cns constituting more than 80% of the newly synthesised proteins) in media and radioactive proteins in cells were quantified (Table 2). After 60 minutes of chase there was a marked decrease (≈56 to 75%) of the proportion of labelled Cns released by explants from α_{S1}-Cn-deficient goats as compared to explants from *α_{S1}-CnA/A* goats. After 120 minutes of chase, this reduction of secretion was maintained (≥55%) in samples from *α_{S1}-CnF/F* and *α_{S1}-CnO/O* goats. It is interesting to note, however, that, at that chase time, Cns secretion was less affected in *α_{S1}-CnE/E* MEC, the proportion of labelled Cns found in the medium being only reduced by ≈25%. These comparisons indicated that the efficiency of Cn secretion was correlated with the relative level of α_{S1}-Cn in milk.

α_{S1}-Cn is required for the optimal export of β-Cn and κ-Cn from the ER

In contrast to β-Cn and κ-Cn, we did not detect an accumulation of immature α_{S1}-Cn in the tissues from *α_{S1}-CnE/E* or *α_{S1}-CnF/F* goats. If α_{S1}-Cn plays a role in the export

Fig. 4. Comparison of the protein content of MEC either producing high amount or lacking α_{S1}-Cn and identification of the main secretory and ER-resident proteins. (A) Aliquots of PNS from *α_{S1}-CnA/A* (A/A) or *α_{S1}-CnO/O* goats (O/O) were analysed by 2D-PAGE followed by Coomassie Blue staining. Numbered spots were subjected to N-terminal sequencing (see Table 1). Estimated isoelectric points (pI) are indicated. SA: serum albumin; Ac: actin; circle, CNX: location of calnexin; CRT: calreticulin; other: see B. (B) Proteins contained in the PNS prepared from *α_{S1}-CnO/O* goat (1:10 of the aliquot used for protein staining) were separated by 2D-PAGE as above, transferred to nitrocellulose, incubated with rabbit antisera against goat α-lactalbumin (LA), κ-Cn (κ-Cn), β-lactoglobulin (LG) or PDI, and detected with HRP-conjugated anti-rabbit IgG followed by ECL. The corresponding Coomassie Blue-stained proteins are identified in A. The major form of κ-Cn matches spot number 4.

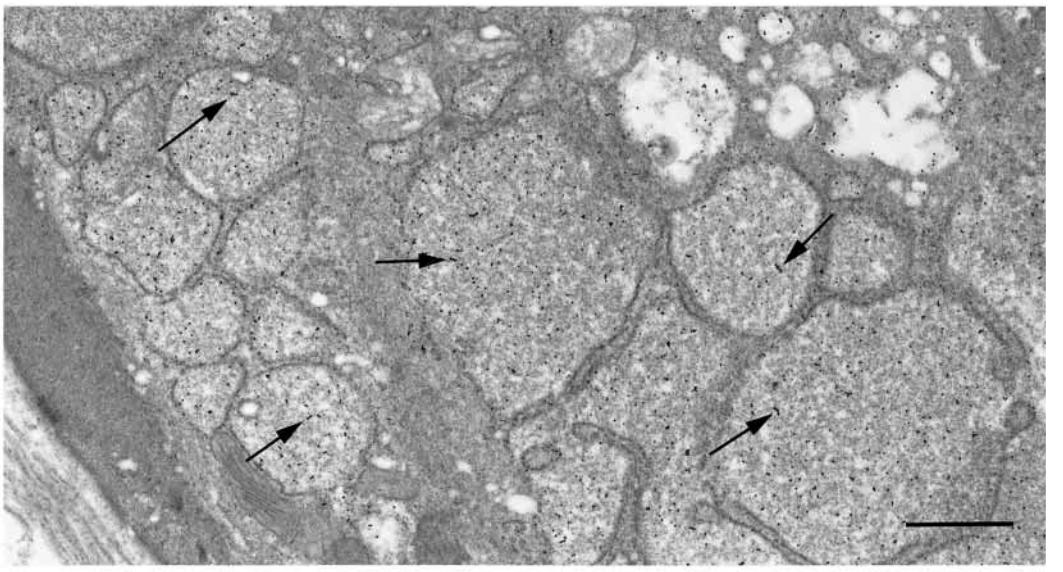

Fig. 5. Immunolgold labelling of *α_{S1}-CnO/O* MEC for κ-Cn. Fragments of the mammary gland of *α_{S1}-CnO/O* goat, fixed and processed for immunoelectron microscopy, were immunogold labelled with antibody against κ-Cn. Note that the labelling is restricted to compartments of the secretory pathway and is over the whole electron dense material in the distended ER cisternae (arrows). Bar, 1 μm.

of the other Cns out of the ER, one can speculate that, whatever the level of expression of α_{S1}-Cn, its kinetic of transport to the Golgi would be fairly constant. Alternatively, accumulation of β-Cn and κ-Cn could reflect their relative overexpression. Hence, we wished to compare the rate of synthesis of the Cns and the kinetics of α_{S1}-Cn export from the ER in MEC from *α_{S1}-CnA/A* and α_{S1}-Cn-deficient goats. Since phosphorylation of the Cns, and O-glycosylation of κ-Cn, occurs in the Golgi apparatus, one means to study the rate of ER to Golgi transport of the Cns is to monitor the acquisition of these post-translational modifications.

For this study, we first had to determine the molecular form of α_{S1}-Cn in the ER. Since α_{S1}-CnA and α_{S1}-CnE differ by 4 amino acid substitutions (^{16}Leu→Pro, ^{77}Gln→Glu, ^{100}Arg→Lys, ^{195}Thr→Ala), we suspected that they would have slightly different behaviours in 2D-PAGE. Therefore Cns prepared from the milk of *α_{S1}-CnA/A* and *α_{S1}-CnE/E* goats were de-phosphorylated using potato acid phosphatase and analysed by 2D-PAGE. Phosphatase treatment of Cns from *α_{S1}-CnA/A* goat milk generated two main spots (Fig. 6, A/A, Phosphatase). Mixing and co-electrophoresis of these products with the proteins prepared from the mammary gland of *α_{S1}-CnO/O* goat (Fig. 6, A/A, Phosphatase+PNS O/O) showed that the more basic polypeptide strictly co-migrated with the molecular form of β-Cn accumulated in the ER of α_{S1}-Cn-deficient goats. The second polypeptide (arrow in Fig. 6, A/A, Phosphatase), which was not detectable in the PNS from *α_{S1}-CnO/O* goat (see Fig. 4A), appeared therefore as an additional spot (Fig. 6, A/A, Phosphatase+PNS O/O, arrow). We concluded that this spot corresponded to the ER form of α_{S1}-Cn encoded by the *A* allele. Similarly, after phosphatase treatment of Cns from *α_{S1}-CnE/E* goat milk (Fig. 6, E/E, Phosphatase) two main spots were found, the more basic proteins corresponding to the immature form of β-Cn. As anticipated above, the second polypeptide (Fig. 6, E/E, Phosphatase; short arrow) had a slightly reduced electrophoretic mobility and was slightly more acidic, as compared to the immature α_{S1}-CnA variant. After mixing with the proteins from the mammary gland of *α_{S1}-CnO/O* goat, this protein also appeared as an additional spot (Fig. 6, E/E, Phosphatase+PNS O/O, short arrow). We concluded that this spot corresponded to the ER form of α_{S1}-Cn encoded by the *E*

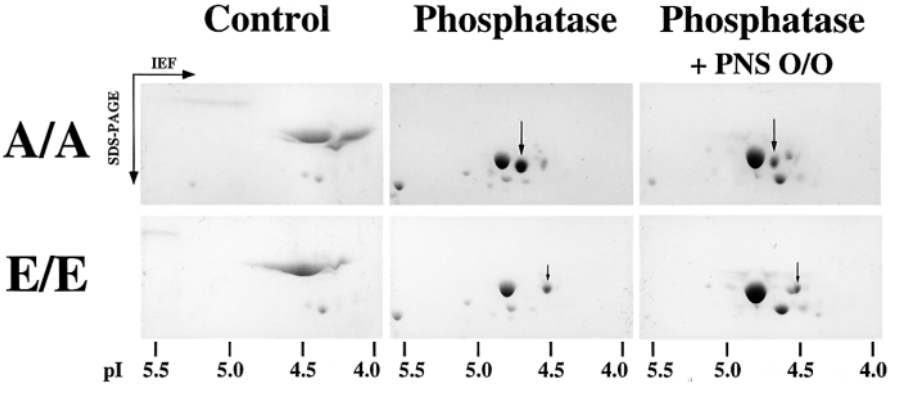

Fig. 6. Identification of the ER form of α_{S1}-Cn in 2D-PAGE gels. Cns fraction from *α_{S1}-CnA/A* (A/A) or *α_{S1}-CnE/E* (E/E) goat milk were incubated either in the absence (Control) or presence of potato acid phosphatase (Phosphatase). In addition, acid phosphatase-treated Cns and proteins contained in the PNS prepared from *α_{S1}-CnO/O* goat were mixed (Phosphatase + PNS O/O). Samples were analysed by 2D-PAGE followed by Coomassie blue staining. Only the area of the gels surrounding the Cns is shown. Estimated isoelectric points (pI) are indicated. Note that phosphatase treatment of each milk Cns leads to the production of a protein (arrows) which is not present in the PNS prepared from the mammary gland of *α_{S1}-CnO/O* goat (compare with Fig. 4, A, O/O). Note also that these additional proteins have distinct electrophoretic mobilities in samples A/A (arrow) and E/E (small arrow). These proteins were identified as α_{S1}-Cn by N-terminal sequencing.

allele. Four lines of evidence further showed that these spots corresponded to immature α_{S1}-CnA/A or α_{S1}-CnE/E. First, after a brief labelling of mammary gland explants with Pro-mix™ (Fig. 7A, Pulse, i_{S1}A and i_{S1}E), there was a strict co-migration of each of the de-phosphorylated α_{S1}-Cn with a radioactive spot. Second, α_{S1}-CnA was not present in MEC from *α_{S1}-CnE/E* goat and vice versa. Third, both proteins were lacking in MEC from *α_{S1}-CnF/F* and *α_{S1}-CnO/O* goats (data not shown). Fourth, the N-terminal sequences of the two proteins (RPKHPIN) were identical and corresponded to that of goat α_{S1}-Cn.

The identification of the mature forms of β-Cn and α_{S1}-Cn on 2D-PAGE was based on the following assumptions. First, these forms should not be labelled after a short pulse with Pro-mix™. Second, mature [^{35}S]methionine/cysteine-labelled β-Cn should appear during chase in MEC from all α_{S1}-Cn

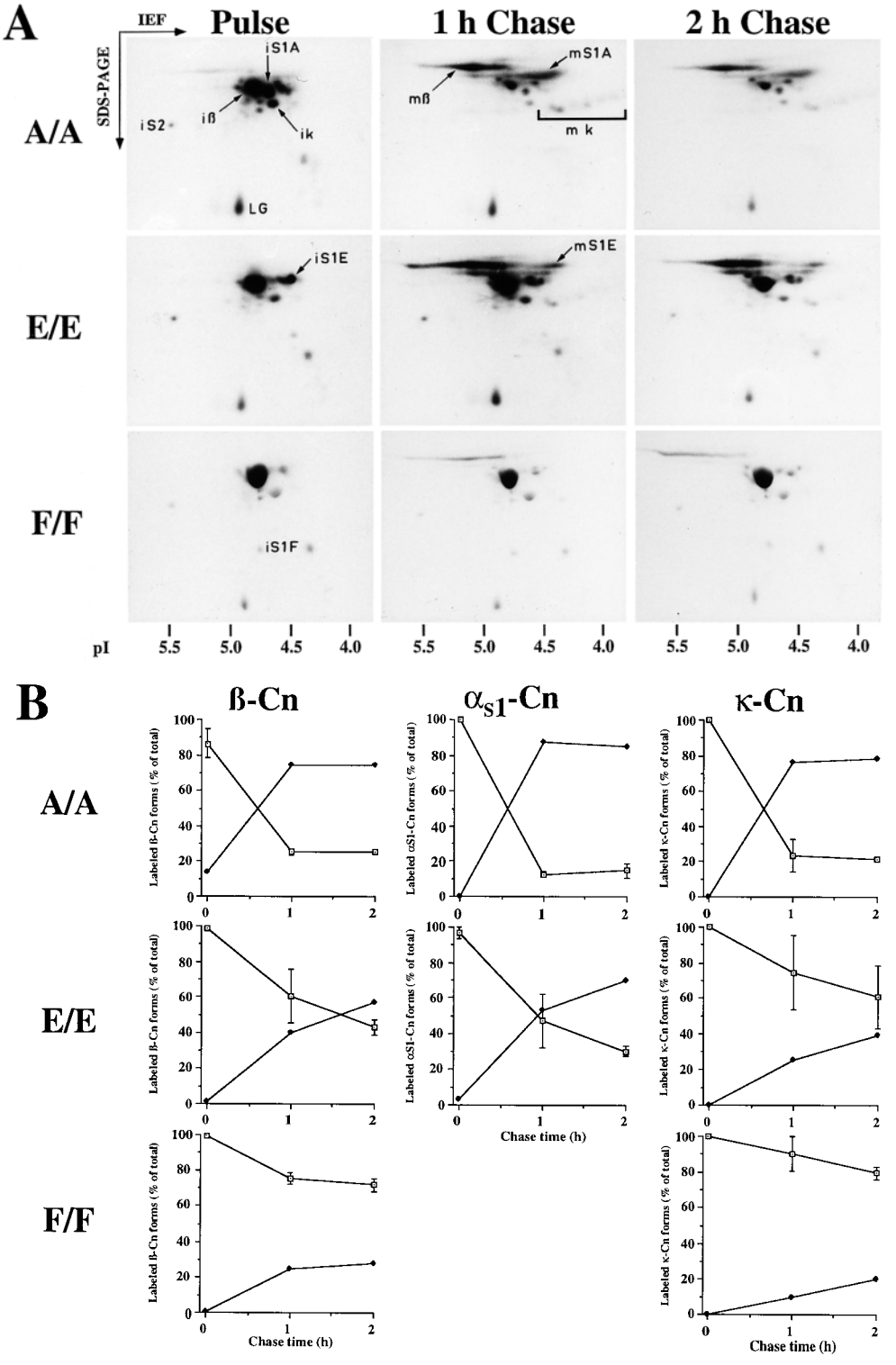

Fig. 7. In α_{S1}-Cn-deficient goats, transport of β-Cn and κ-Cn from the ER to distal compartment of the secretory pathway is slowed down. Mammary gland explants from the indicated animals were pulse-labelled for 5 minutes with Pro-mix™ and chased for 0 (Pulse), 1 (1 h Chase) or 2 hours (2 h Chase). At each chase time point, tissues were collected and [^{35}S]methionine/cysteine-labelled proteins were analysed by 2D-PAGE followed by fluorography.
(A) Fluorograms. Only the area of the fluorograms surrounding the Cns is shown. Estimated isoelectric points (pI) are indicated. i_{S1}A: immature α_{S1}-CnA; iβ: immature β-Cn; i_{S2}: immature α_{S2}-Cn; iκ: immature κ-Cn; LG: β-lactoglobulin; m_{S1}A: mature α_{S1}-CnA; mβ: mature β-Cn; mκ: mature κ-Cn; i_{S1}E: immature α_{S1}-CnE; m_{S1}E: mature α_{S1}-CnE; i_{S1}F: immature α_{S1}-CnF. Note the identical electrophoretic mobilities of proteins i_{S1}A and i_{S1}E with the proteins identified in Fig. 6 by an arrow or a short arrow, respectively.
(B) Quantification of the molecular forms of the Cns. For the indicated Cns, except α_{S1}-CnF, the total (immature plus mature) [^{35}S]methionine/cysteine-labelled Cns at each time point was calculated for the various goat variants. For each condition, the labelled immature (open square) or mature (closed circle) form of the Cns is expressed as a percentage of the total. The mean of duplicate experiments is shown. Bars (only shown for immature forms) indicate the variation of the single values from the mean.

variants whereas a labelled spot co-migrating with mature α_{S1}-Cn should not emerge in *α_{S1}-CnF/F* and *α_{S1}-CnO/O* MEC, at any chase time. The proteins labelled m_{S1}A, m_{S1}E and mβ in Fig. 7A fulfilled all these criteria. Identification of the mature form of κ-Cn as the multiple acidic spots labelled mκ was done by immunoblotting (data not shown).

Having identified the immature and mature forms of the main Cns on 2D-PAGE, we evaluated their maturation. Consistent with the relative proportion of β-Cn in goat milk, we observed that, after a 5 minutes pulse, the main [^{35}S]methionine/cysteine-labelled protein in MEC from *α_{S1}-CnA/A* goat was immature β-Cn (Fig. 7A, iβ). In MEC from *α_{S1}-CnE/E* goat, newly synthesised β-Cn was found in higher amount whereas a substantial decrease of the relative amount of [^{35}S]methionine/cysteine-labelled α_{S1}-Cn and κ-Cn was observed (Fig. 7A, i_{S1}E and iκ). This increase in β-Cn synthesis, which was also observed in *α_{S1}-CnF/F* goat, was clearly superior to the decrease of α_{S1}-Cn. This was in agreement with our observation that the overall incorporation of radioactivity was higher in explants prepared from *α_{S1}-CnE/E* goats (data not shown). These data also indicated that the massive accumulation of the Cns in the ER of α_{S1}-Cn-deficient animals does not inhibit protein synthesis. Comparison of the maturation of each of the main Cns (Fig. 7B) revealed that in *α_{S1}-CnA/A* MEC, conversion of all newly synthesised Cns to their mature forms, i.e. their transport to the Golgi apparatus, was virtually complete (≥75-85%) as from 1 hour chase. In *α_{S1}-CnE/E* MEC, conversion of all immature Cns was slowed down but, interestingly, the maturation of α_{S1}-Cn (≈70% mature form after 2h chase) was less affected than that of both β-Cn and κ-Cn (at most ≈55%). In *α_{S1}-CnF/F* MEC only ≈20-30% of the mature form of β-Cn and κ-Cn were observed after 2 hours chase. It is noteworthy that, over this period of chase, significant degradation of the Cns which accumulated in α_{S1}-Cn-deficient MEC was not observed.

These comparisons demonstrated that the decrease in Cns secretion observed in α_{S1}-Cn-deficient goats was largely due to a decrease in the export of both β-Cn and κ-Cn from the ER and, most importantly, indicated that the efficiency of their transport to the Golgi apparatus was dependent upon the relative amount of α_{S1}-Cn in the ER. These results strongly suggested that α_{S1}-Cn would play a key role in this transport step.

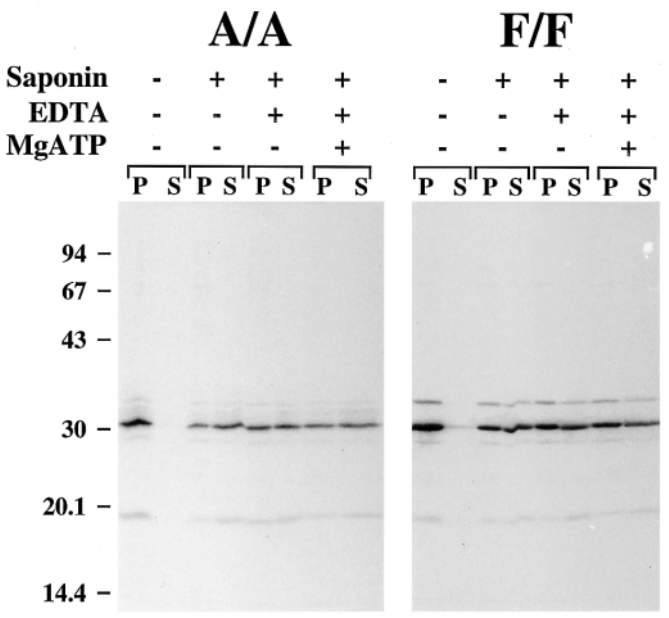

Fig. 8. Cns which accumulate in MEC from α_{S1}-Cn-deficient goats are released from saponin-permeabilised ER vesicles. [^{35}S]methionine/cysteine-labelled ER vesicles were sedimented from the PNS prepared from mammary gland explants from *α_{S1}-CnA/A* (A/A) or *α_{S1}-CnF/F* (F/F) goats pulse-labelled for 5 minutes with Pro-mix™, resuspended in release buffer and incubated in the absence (–) or presence (+) of saponin, EDTA or EDTA plus MgATP. After centrifugation, pellet (P) and supernatant (S) were analysed by SDS-PAGE followed by fluorography. Positions of the molecular mass markers (kDa) are indicated on the left.

Release of accumulated Cns from the lumen of the ER upon permeabilisation of the membranes by saponin

It has been shown that proteins which accumulate in the ER may spontaneously form aggregates. In some instances proteins in such aggregates are disulphide-bonded (Marquardt and Helenius, 1992; Tooze et al., 1989). The possibility that all accumulated Cns would be disulphide cross-linked in the ER of MEC from α_{S1}-Cn-deficient goats is very unlikely because goat β-Cn lacks cysteine. To test the solubility of the Cns accumulated in the ER, [^{35}S]methionine/cysteine-labelled ER vesicles sedimented from the PNS prepared from pulse-labelled mammary gland explants were permeabilised with saponin. This treatment was previously shown to allow the release of soluble luminal proteins but not large aggregates (Chanat and Huttner, 1991). Moreover, since at least β-Cn could theoretically be induced to aggregate by the calcium contained in the ER (Sambrook, 1990), we analysed the effect of EDTA on the release of the Cns. Finally, given the observation that BiP was increased in α_{S1}-Cn-deficient goats, and therefore may be involved in Cn retention in the ER, the effect of MgATP, which is known to release BiP from misfolded proteins or unassembled subunits retained in the ER (Hurtley et al., 1989), was also studied. Upon permeabilization of the membranes of ER vesicles obtained from *α_{S1}-CnA/A* MEC (Fig. 8, A/A), we recovered ≈55-60% of the [^{35}S]methionine/cysteine-labelled immature forms of the Cns in the supernatant. The protein with M_r of ≈19000, which correspond to β-lactoglobulin, followed a similar distribution between pellet and supernatant. The fact that a substantial proportion of these proteins, as well as PDI (data not shown), remained in saponin treated ER vesicles was consistent with previous results (Chanat and Huttner, 1991). We think that this reflects the incomplete permeabilization of the ER membranes due to its low cholesterol content. Interestingly, no significant decrease in the release of immature β-Cn and κ-Cn was observed after saponin permeabilization of the ER vesicles prepared from *α_{S1}-CnF/F* (Fig. 8, F/F) or *α_{S1}-CnE/E* MEC (data not shown). Finally no increase in the release of the Cns was observed in the presence of either EDTA or EDTA plus MgATP, whatever the source of ER vesicles. Taken together, these results strongly suggested that the Cns which accumulate in the ER of MEC from α_{S1}-Cn-deficient goats are in a soluble form.

Accumulation of β-Cn in the RER of hormone-treated HC11 cells

We investigated whether the lack of α_{S1}-Cn could also result in ER accumulation of the Cn in another cell system, the mouse MEC line HC11. In agreement with previous studies (Ball et al., 1998), immunofluorescence of control and hormone-treated HC11 cells revealed that only ≈30 to 40% of the cells expressed milk proteins in response to PRL (Fig. 9A). Interestingly, the immunostaining was found in the whole cytoplasm. Consistent with this, EM analysis revealed that the RER of hormone-treated HC11 cells was distended and filled with a granular electron dense material (Fig. 9B). Immunoblotting revealed that β-Cn, but not α-Cns which in mouse have M_r of ≈40000, was expressed in our conditions of culture (Fig. 9C). Only trace amount of β-Cn, however, was found in the apical medium (Fig. 9C, Med, A), even after 3 hours incubation (data not shown). On the other hand, we noted that cell-associated β-Cn (Fig. 9C, Cell, +) was as a doublet of faster electrophoretic mobility than the mature β-Cn (M_r ≈30000) detected in mouse milk (Fig. 9C, M), these molecular forms most likely corresponding to immature forms of β-Cn. We conclude from these results that, similarly to what was observed in MEC from α_{S1}-Cn-deficient goat, β-Cn accumulated in the RER of HC11 cells which do not express α_{S1}-Cn, suggesting that the involvement of α_{S1}-Cn in the transport of other Cns from the ER is not restricted to goat MEC.

DISCUSSION

Morphological studies have led to the conclusion that formation of Cn submicelles would occur after the export of the Cns from the ER (Clermont et al., 1993) and proceed into Cn micelles in the late secretory pathway, via calcium-mediated aggregation subsequent to their phosphorylation in the Golgi apparatus. However our data strongly suggest that interactions between the Cns might already take place in the ER. We have shown that, in MEC from goats which do not express α_{S1}-Cn, the rate of transport of β-Cn and κ-Cn out of the ER is considerably slowed down. In contrast, in MEC which do not express β-Cn, we obtained evidence that Cns transport is virtually unaffected. This was consistent with the finding that the knock-out of the *β-Cn* gene in mouse had no significant effect on Cns secretion (Kumar et al., 1994). These results demonstrate that α_{S1}-Cn has a specific role to play in the secretion of the Cns. We also observed that the level of

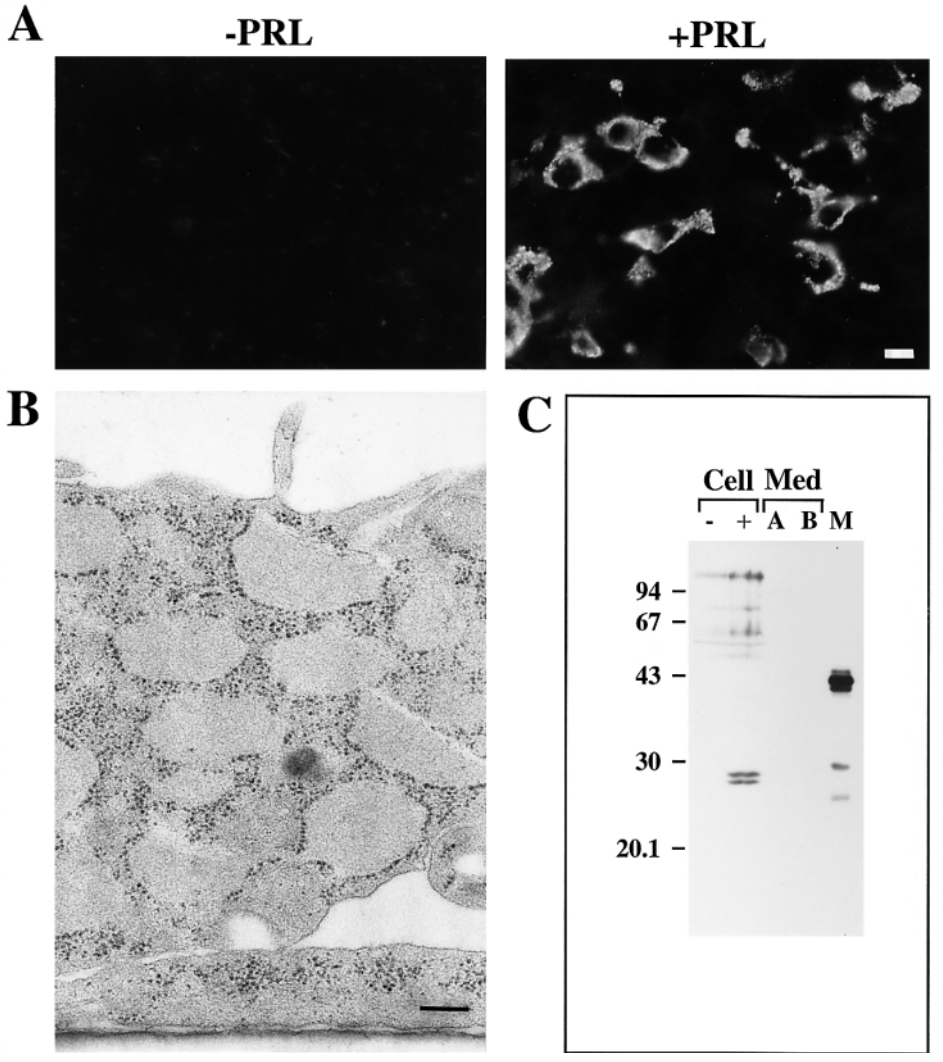

Fig. 9. Accumulation of β-Cn in distended RER cisternae of PRL-treated HC11 cells. (A) Induction of Cn synthesis in HC11 cells upon PRL treatment. Filter grown HC11 cells were cultured in the absence (–PRL) or presence of PRL (+PRL) for 7 days and subjected to immunofluorescence using rabbit antiserum against mouse Cn #7781. Bar, 10 μm. (B) Morphology of PRL-treated HC11. After induction of Cns synthesis by PRL, filter-grown HC11 cells were fixed and processed for EM. Note the distended cisternae of the RER filled with a granular electron-dense material. Bar, 0.2 μm. (C) Lack of α_{S1}-Cn synthesis and β-Cn transport in PRL-treated HC11 cells. Control (–) and PRL-treated (+) HC11 cells were further incubated for 1 hour in fresh serum-free medium in the absence or presence of PRL, respectively. Cell extracts, apical and basolateral media were collected. One tenth aliquot of the cells' extracts (Cell), complete apical (A) and basolateral (B) media (Med) from PRL-treated cells, and an aliquot of crude mouse milk (M) were analysed by SDS-PAGE followed by immunoblotting, using a rabbit antiserum against mouse milk specific proteins (RAM/MSP) and ECL. Note that the β-Cn present in PRL-treated cells is as a doublet of faster electrophoretic mobility than β-Cn from mouse milk. The identification of this doublet as β-Cn was shown by immunoblot using a β-Cn specific antiserum (data not shown). Positions of the molecular mass markers (kDa) are indicated on the left.

accumulation of the Cns in the RER was proportional to the level of α_{S1}-Cn in this compartment, which is consistent with α_{S1}-Cn exerting a positive role in the transport of the other Cns to the Golgi apparatus. Moreover, the rate of transport of α_{S1}-Cn out of the ER was apparently almost independent of its level of expression. We conclude that α_{S1}-Cn is required for the optimal transport of the all Cns from the ER to the Golgi apparatus.

Cn micelle formation in MEC from β-Cn- or α_{S1}-Cn-deficient goats

Since Cns aggregate during their journey in the secretory pathway, one can hypothesize that modification of the Cn content of MEC would perturb Cn micelle formation. However, submicelles and micelles were still observed in the late secretory pathway of MEC which do not express β-Cn or from α_{S1}-Cn-deficient goats, confirming the results obtained with β-Cn-deficient mice (Kumar et al., 1994). This demonstrates that neither Cn is absolutely necessary for the formation of the Cn micelle. On the other hand, it is not surprising that Cn micelle formation still occurs in the absence of certain Cns. The formation of the micelle is essentially based on hydrophobic and electrostatic interactions between Cns. This is achieved by aggregation of the Cn after neutralization of their negative charges, due to their abundant acidic residues, by calcium and formation of electrostatic bridges between the clusters of serine phosphate contained in the calcium sensitive Cns by colloidal calcium phosphate. Any Cn that reaches the Golgi apparatus might therefore be able to aggregate, at least with itself. Self-association reactions of each Cn, or of individual Cn with the other Cns, have been extensively studied and it has been known for a long time that micellar aggregates can be reconstituted in vitro (for review see Holt, 1992; Rollema, 1992).

ER quality control and accumulation of the Cns in the ER of α_{S1}-Cn-deficient MEC

Accumulation of secretory proteins in the ER has been observed in a certain number of both exocrine and endocrine cell systems in which the synthesis of secretory proteins was hyper stimulated (e.g. Tooze et al., 1989 and references therein; Noda and Farquhar, 1992). Another well-known case proceeds from the blockage of the export of newly synthesised proteins from the ER. Proper folding or oligomerisation of secretory proteins is often a prerequisite for their forward transport from the ER. For hetero-oligomeric structures, default in oligomerisation could be due to either misfolding or the lack of one of the partners. This process, which ensures that only proteins which have achieved their correct conformation are exported from the ER, has been called ER quality control (for review see Hammond and Helenius, 1995).

The simplest explanation of our finding that, in α_{S1}-Cn-deficient MEC, the other Cns are retained in the ER, is that formation of an hetero-oligomeric complex between the Cns is required for their efficient packaging in ER-derived transport vesicles and their optimal forward transport to the Golgi. The observation that this transport step was not significantly affected in the case of β-lactoglobulin and α-lactalbumin, two whey proteins which are not known to interact with Cns, was consistent with this possibility. Creation of this oligomeric structure, however, would not be equally demanding for each of the Cns since Cn trafficking in β-Cn null MEC appeared not to be affected. We propose that α_{S1}-Cn plays a crucial role in the formation and/or transport of a Cn complex which would form in the ER. Such a role has been demonstrated, for example, for the α-subunit of the glycoprotein hormones which dimerises with the hormone specific β-subunit in the ER (Hoshina and Boime, 1982). It is not clear whether this primary complex of Cns coincides with the Cn submicelle because these structures were only observed as from the cis-Golgi (Clermont et al., 1993).

It has been shown that secretory proteins which undergo increased concentration in the ER may spontaneously form aggregates. For example in the exocrine pancreas, the pancreatic digestive enzymes co-aggregate in the RER into the so-called intracisternal granules, upon induction of their synthesis (Tooze et al., 1989). In this study, we have obtained several lines of evidence that accumulated Cns fail to form large aggregates. First, permeabilization of membranes by saponin at slightly basic pH and at physiological ions concentration allowed the release of an identical amount of newly synthesised immature Cns from the ER of both control and α_{S1}-Cn-deficient MEC. Second, the release of β-lactoglobulin, a whey protein which is not known to interact with Cns, was similar to that of the Cns. These results argue against the possibility that inhibition of Cns export out of the ER might be due to the formation of insoluble aggregates which would be unable to be packaged into the ER transport vesicles.

Finally, in response to the accumulation of unfolded proteins in the ER, the synthesis of several ER-resident proteins is selectively induced (Kim et al., 1996; Kozutsumi et al., 1988; Macer and Koch, 1988). The signalling pathway responsible for this induction has been named the unfolded-protein response (for review see Pahl and Baeuerle, 1997). Accumulation of the Cns in the ER of α_{S1}-Cn-deficient MEC also resulted in an increase of the amounts of GRP 94, BiP, calreticulin, and PDI. To ascertain whether any of these ER-resident proteins was involved in the retention of the Cns in the ER, we investigated whether the synthesis of some of them was induced. These experiments were hampered by the fact that, except for BiP, very few labels incorporated into these proteins after long-term labelling (data not shown), in a manner that did not allow reliable quantitation of their rate of synthesis. This was most likely due to the fact that (1) those proteins have a very slow turn over and (2) steady state level of these proteins was reached at the time of the experiments. Consistent with this later hypothesis, the synthesis of BiP was not significantly increased in the tissues from α_{S1}-Cn-deficient goats. These results and the fact that at least some of the ER-resident proteins mentioned above are not very likely to interact with Cns accumulated in the ER (see below) suggest that their increase in α_{S1}-Cn-deficient MEC may rather correspond to the increased volume of the ER in these cells. For example, it is conceivable that, in distended ER cisternae, an increased amount of calreticulin is necessary for the maintenance of calcium homeostasis. Also, it has been postulated that the cellular level of PDI would reflect the size of the RER (see Kim et al., 1996, and references therein).

Possible role of α_{S1}-Cn in forward transport of the Cns out of the ER

It has been proposed that newly synthesised secretory proteins

move to the Golgi by bulk flow (Wieland et al., 1987), i.e. that no specific signal is required for their forward transport out of the ER. If we accept this proposal, retention of β-Cn and κ-Cn in the ER of α_{S1}-Cn-deficient MEC would be via interaction with ER chaperones. The association of α_{S1}-Cn with the other Cns in an oligomeric complex, most likely through their hydrophobic domains, would bury these hydrophobic determinants which are the usual targets of ER chaperones such as BiP, allowing forward transport of the Cns complex to the Golgi. In this study, however, we did not obtain evidence of the binding of BiP to accumulated Cns. Of course, Cns might be retained in the ER by interacting with other chaperones. Calreticulin, which is also up regulated in α_{S1}-Cn-deficient MEC, is not a good candidate since neither of the Cns has N-linked carbohydrates, except if, as was shown for calnexin (Kim and Arvan, 1995), it also has the capability to interact with non-glycosylated proteins. Finally, one would have to postulate that the machinery for retention would be saturated since Cns are not completely blocked in the ER, even in MEC which lack α_{S1}-Cn.

An alternative model for the selective export of secretory proteins from the ER has recently emerged. In this model it is postulated that at least some secretory proteins would be concentrated into transport vesicles for their efficient transport to the Golgi (Balch et al., 1994; Kuehn and Schekman, 1997; Mizuno and Singer, 1993). A correlative to this model is that a sorting receptor is required for the selective packaging of soluble secretory proteins into ER transport vesicles. This also implies that cargo proteins contain sorting information. A similar mechanism has been implicated at the exit site of the TGN in the sorting of secretory proteins to the regulated secretory pathway (Burgess and Kelly, 1987). However, searches for the receptors involved in this sorting step have been unsuccessful, as have been efforts to identify anterograde sorting motif and putative sorting receptors at the ER level. Nevertheless, a sorting signal to the regulated secretory pathway has been identified in chromogranin B (Chanat et al., 1993), a member of the granin family of proteins that are found in secretory granules in a wide variety of endocrine cells and neurons. Moreover, a fraction of chromogranin B was shown to be tightly associated with membranes of the secretory pathway and this interaction has been proposed to be involved in the sorting process (Pimplikar and Huttner, 1992). In this context, our results suggest that α_{S1}-Cn would contain the sorting information for the efficient anterograde transport of the Cns out of the ER.

In support to these two possibilities is the observation that transport of the Cns is correlated with the relative proportion of α_{S1}-Cn in the ER. By virtue of interacting with the other Cns in an hetero-oligomeric complex, αS1-Cn would therefore promote the transport of all the Cns to the Golgi.

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