ENZYMATIC PROCESSING OF PRECURSORS OF OVINE LACTOPROTEINS BY MAMMARY MICROSOMAL MEMBRANES AND A DEOXYCHOLATE-SOLUBLE EXTRACT FROM ROUGH MICROSOMES

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1. Introduction

The signal hypothesis first proposed in [1] and further refined [2,3] has provided an attractive model to explain adequately the mechanism involved in selecting specific mRNAs for translation on bound polyribosomes at the early stage of protein secretion. Analyses of miscellaneous secretory proteins synthesized in various cell-free systems have shown that the polypeptide chains thus manufactured contain hydrophobic amino terminal extensions containing up to 30 amino acid residues [4,5]. This hydrophobic segment, called the 'signal', interacts with the endoplasmic reticulum (ER) membranes, thus providing the topological conditions for the vectorial discharge of the nascent polypeptide chain into the ER lumen. Subsequently the 'signal' is selectively removed through proteolytic cleavage, which does occur before completion of the chains, since the processing of preproteins can only be achieved when microsomal membranes are already present in the cell-free translation system [2,3,6–11].

In order to investigate the mechanism of protein secretion in the lactating mammary gland, the primary structures of the 6 major lactoproteins (α_{s1}-, α_{s2}-, β- and κ-caseins, β-lactoglobulin and α-lactalbumin) synthesized in a cell free system were analysed. The in vitro translation products were found to contain amino terminal extensions of 15 amino acid residues for the first three caseins and of 21, 18 and 19 for the three latter proteins, respectively [5,12,13].

We demonstrate here that the prelactoproteins synthesized in a cell-free system in the presence of mammary microsomal membranes are transferred into the lumen of the microsomal vesicles and processed into authentic lactoproteins as indicated by amino terminal sequence analyses. Furthermore, we have shown that the signal peptide can be post-translationally removed by protease(s) extracted from rough mammary microsomes by sodium deoxycholate (DOC) in agreement with the data in [14].

2. Materials and methods

2.1. *Isolation of rough microsomes from mammary gland*

Post-mitochondrial supernatant in 0.25 M sucrose in TKM buffer (20 mM Tris–HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$) was prepared from lactating mammary gland as in [15]. This supernatant was layered over a step gradient consisting of 2 M and 1.7 M sucrose in TKM buffer and centrifuged for 20 h at 135 000 × *g* at 2–4°C. Rough microsomes were collected from 1.7/2 M sucrose interface, diluted with 2 vol. TKM buffer and sedimented through a cushion of 1.3 M sucrose for 1 h at 170 000 × *g*. The pellets were stored at −70°C or used immediately.

2.2. *Preparation of degranulated microsomal membranes*

The procedure used was essentially as in [3]. Fresh

pellets of rough microsomes were resuspended by gentle homogenization in 20 mM Tris–HCl (pH 7.4), 50 mM KCl and EDTA was added to 2 μmol/10 A_{260} units rough microsomes final conc. This suspension was layered over a preformed sucrose gradient containing (0.5, 1.0, 1.3, 1.5, 1.7 and 2 M) sucrose in TKM buffer (20 mM Tris, 50 mM KCl, 0.5 mM DTE) and centrifuged for 4 h at 80 000 × *g*. The degranulated membranes which band at the 1.3/1.5 M sucrose interphase were collected, diluted with TKM buffer and centrifuged at 115 000 × *g* for 1 h. The pellets were resuspended in 20 mM Hepes (pH 7.5), 50 mM KCl 2 mM Mg acetate, 1 mM DTE to 40–50 A_{260} units/ml and stored at −70°C until use.

2.3. *Preparation of the detergent extract*

Rough endoplasmic reticulum or degranulated membranes were resuspended by homogenization in TKM buffer to 50 A_{260} units/ml, then DOC or Triton X-100 was added to 0.5% and 1% final conc., respectively. The resulting clear suspension was centrifuged 1 h at 180 000 × *g*. The peptidase activity of the resulting supernatant was measured either immediately or after storage at −180°C.

2.4. *Translation of mRNA*

Translations were performed using a wheat germ cell-free system as in [12] or reticulocyte lysates treated with staphylococcal nuclease to reduce endogenous globin mRNA activity [15]. Degranulated microsomal membranes were added in varying amounts and the incubation was carried out at 26°C for 90 min. When translation was carried out in the presence of f-[^{35}S]Met-tRNA$_f$, unlabeled methionine (50 μM) was added into the reaction medium, to prevent the incorporation of the [^{35}S]Met released from the added tRNA.

The preparation of immunoprecipitates for polyacrylamide gel electrophoresis and sequence analyses were as in [12].

2.5. *Post-translational cleavage of preproteins*

The solubilized peptidase activity was measured in cell-free translation mixture containing preproteins doubly labeled with [^{35}S]methionine provided by f-[^{35}S]Met-tRNA$_f$ and [^{3}H]amino acids. Aliquots of detergent extract (2–10 μl) in 100 μl total vol. (containing 20–50 μl cell-free translation mixture) were incubated at 25°C during various periods and the radioactivity was measured on immunoprecipitates.

2.6. *Sources of material*

[^{35}S]Met (600), [^{3}H]Arg (36), [^{3}H]Glu (21), [^{3}H]Gly (10), [^{3}H]Ile (10) were obtained from CEA, France. Numbers in parentheses refer to specific activity in Ci/mM. Sodium deoxycholate was purchased from Sigma Chemical Co., St Louis USA and staphylococcal nuclease from Boehringer, France.

3. Results

3.1. *Cotranslational cleavage of lactoproteins by mammary microsomal membranes*

The addition of degranulated mammary microsomal membranes inhibited the translation of mammary mRNAs by ~30–40% in a wheat germ cell-free system; in contrast, the rate of protein synthesis was not affected in the presence of membranes when translation was carried out in a reticulocyte cell-free system.

Slab gel analysis of the translation products of mammary mRNAs (fig.1, lane 1) showed a major radioactive band which corresponded to a mixture of pre-caseins [12], and a discrete band which was identified as pre-β-lactoglobulin [5]. Pre-α-lactalbumin, which represents only 2% of the total amount of lactoproteins was not detectable on this autoradiogram. When translation was performed in the presence of membranes, the electrophoretic mobility of the major band was found to be slightly reduced (fig.1, lane 2), whereas the discrete band migrated faster (fig.1, lane 2). The analysis of specific immunoprecipitates of β-casein and β-lactoglobulin synthesized in the absence (fig.1, lanes 3,5) and in the presence of membranes (fig.1, lanes 4,6) shows more clearly the difference in the electrophoretic behavior of these proteins.

Protease treatment of the translation mixture was used to study the segregation of prelactoproteins synthesized 'in vitro'. As expected and as shown for other secretory proteins [7,9], prelactoproteins were segregated by microsomal membranes as demonstrated by their resistance to trypsin and chymotrypsin treatment (fig.1, lane 7). In contrast, these proteins were found to be completely digested by protease treat-

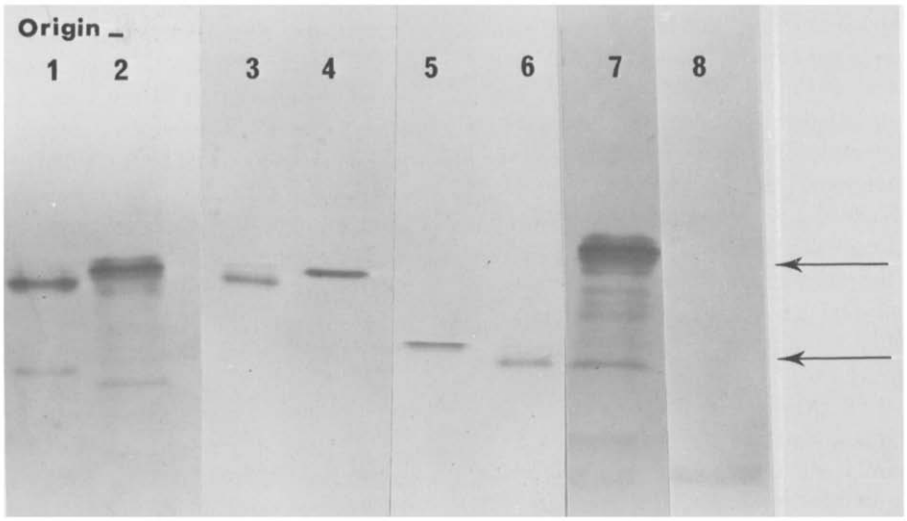

Fig.1. Analysis, by polyacrylamide gel electrophoresis in SDS and subsequent autoradiography, of polypeptides synthesized by translation of ewe mammary mRNAs in a wheat germ cell free system (1) Total ovine mammary mRNA translation products. (2) Translation products synthesized in the presence of microsomal membranes. (3,5) Immunoprecipitates of β-casein and β-lactoglobulin synthesized in the absence of microsomal membranes. (4,6) as (3,4) except that translation was carried out in the presence of membranes. (7) as (2) but the electrophoresis was preceded by digestion with trypsin and chymotrypsin (500 μg/ml) for 90 min at 4°C. (8) as (1) except that the protease treatment was performed before electrophoresis. The arrows indicate the position of authentic β-casein and β-lactoglobulin. Lanes were derived from 3 differents slab gels and aligned according to the positions of 2 proteins markers (authentic β-casein and β-lactoglobulin) on each slab gel.

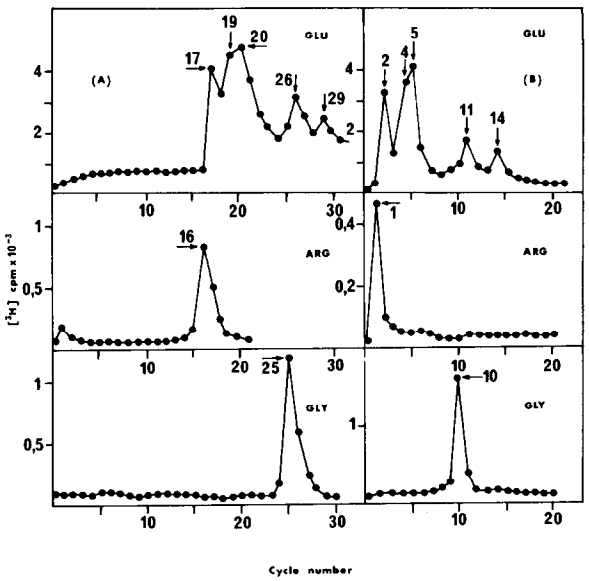

Fig.2. Radiosequence analyses of pre-β-casein synthesized in a wheat germ cell-free system in the absence (A) and the presence (B) of mammary microsomal membranes. The translation of casein mRNAs isolated from bound polysomes of a lactating ewe mammary gland, the immunoprecipitation of pre-β-casein and its microsomal membrane-treated counterpart as well as the radiosequence analyses of the immunoprecipitates were by the methods detailed in [12]. Numbers written above and below the sequence of pre-β-casein indicate the positions of various amino acid residues in the polypeptide chain of pre-β-casein and authentic β-casein, respectively. The site of cleavage, indicated by an arrow, was deduced from the above data:

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                1
Met–Lys–Val–Leu–Ile–Leu–Ala–Cys–Leu–Val–Ala–

                    15  |  16  17      19  20
Leu–Ala–Leu–Ala–    |  Arg–Glu–Gln–Glu–Glu–Leu–
                    ▼   1   2       4   5
                25  26          29
Asn–Val–Val–Gly–Glu–Thr–Val–Glu–Ser–Leu–...
                10  11          14
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ment when proteolytic digestion was carried out on the polypeptides synthesized in the absence of membranes (fig.1, lane 8) or in the presence of detergent (data not shown).

These results indicate clearly that the lactoproteins synthesized in the presence of the membranes were segregated into microsomal vesicles but a major question, however, was whether or not the resulting polypeptides were the expected authentic lactoproteins. For this purpose, partial amino terminal radiosequence analyses were carried out on β-casein and β-lactoglobulin synthesized in a cell-free system in the absence (fig.2A,3A) and in the presence of membranes (fig.2B,3B), respectively. The data presented in fig.2,3 demonstrated clearly that the enzyme(s) bound to mammary ER membranes can accurately cut off 'signal peptides' from nascent prelactoproteins.

3.2. *'In vitro' conversion of completed pre-β-casein into authentic β-casein by a detergent extract of mammary microsomal membranes*

The following experiments were designed to characterize the enzymatic fraction responsible for the 'signal peptide' removal. Mammary microsomal membranes were disrupted either by Triton X-100 or DOC. The soluble fractions were assayed on prelactoproteins synthesized in the presence of f-[^{35}S]Met-tRNA$_f$ and [^{3}H]Glu, which provided an opportune substrate to examine the specificity of solubilized peptidase(s). The 'signal peptide' devoid of any glutamyl residue contains the unique [^{35}S]Met residue at its amino terminus, whereas the segment corresponding to the authentic lactoproteins contains the whole set of [^{3}H]glutamyl residues. When such radiolabeled prelactoproteins were incubated with either DOC or Triton X-100 soluble extract of mammary membranes, only the former was found to contain a peptidase activity as depicted in fig.4. The ^{35}S content of pre-β-casein was found to decrease as function of time in sharp contrast with the steady ^{3}H content, thus indicating that 'the signal' region was the target of an enzymatic attack. To rule out any involvement of an aminopeptidase in this process the final product was analysed by automated Edman degradation. The radiosequence data clearly revealed the occurrence of authentic β-casein (fig.5) and demonstrated that >10% of

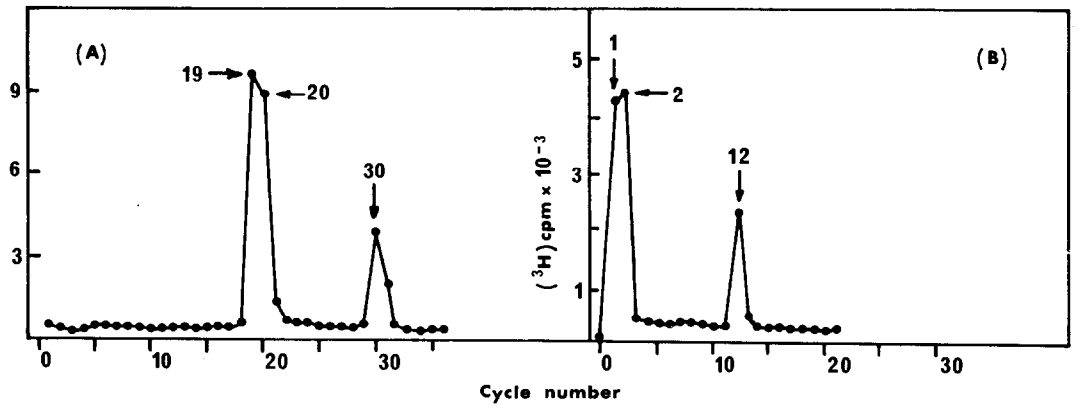

Fig.3. Radiosequence analyses of pre-β-lactoglobulin synthesized in a reticulocyte cell-free system in the absence (A) and the presence (B) of mammary microsomal membranes. Numbers written above and below the sequence of pre-β-lactoglobulin indicate the positions of isoleucine residues in the polypeptide chains of pre-β-lactoglobulin and authentic β-lactoglobulin respectively. The site of cleavage is indicated by arrow:

Met–Lys–Cys–Leu–Leu–Leu–Ala–Leu–Gly–Leu–Ala–Leu–Ala–Cys–Gly–Val–Gln–

19 20 30
Ala–↓ Ile–Ile–Val–Thr–Gln–Thr–Met–Lys–Gly–Leu–Asp–Ile–...
1 2 12

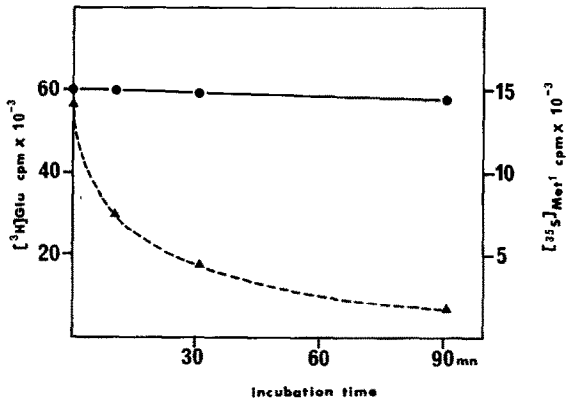

Fig.4. Time-course of proteolysis of pre-β-casein incubated with a DOC-soluble extract from mammary microsomal membranes. The lactoproteins synthesized in a wheat germ cell-free system in the presence of [^{3}H]Glu and f-[^{35}S]Met-tRNA$_f$ were subsequently incubated at 25°C with a DOC-soluble extract from microsomal membranes. Aliquots were collected at various times and treated with antibodies against β-casein. The ^{35}S and ^{3}H contents of the resulting immunoprecipitates were measured by using a dual-label program. The decrease of the ^{35}S content indicates that the 'signal peptide' of pre-β-casein was the target of an enzymatic attack. [^{3}H]Glu (●—●—●); amino terminal [^{35}S]Met (▲- - -▲- - -▲).

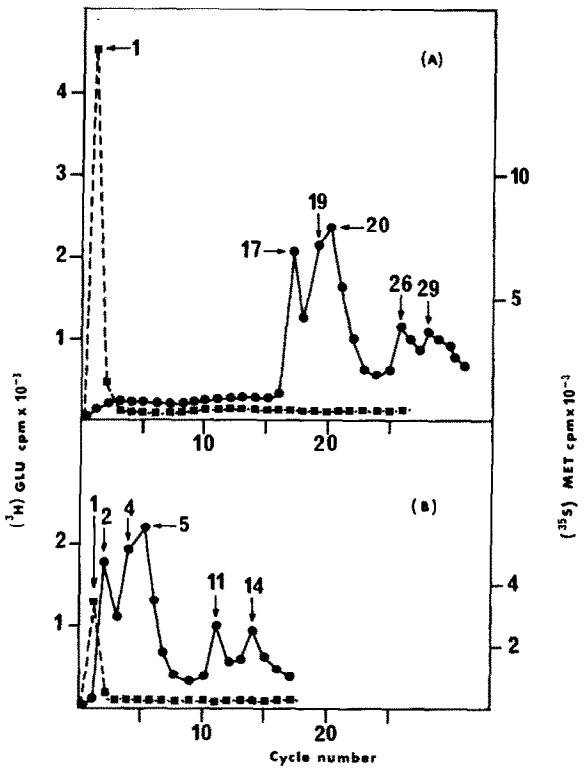

Fig.5. Radiosequence analyses of pre-β-casein (labeled with both [^{3}H]Glu and [^{35}S]Met provided by f-[^{35}S]Met-tRNA$_f$) carried out before (A) and after (B) its incubation with the DOC-soluble extract. The cleaved product in (B) is obviously authentic β-casein according to its partial sequence. The [^{35}S]Met counts at position 1 in panel (B) are presumably due to the presence of uncleaved pre-β-casein. [^{3}H]Glu (●—●); amino terminal [^{35}S]Met (■- - -■- - -■).

pre-β-casein were converted to authentic β-casein by the DOC-soluble peptidase. Similar results were obtained with pre-α_{s1}-casein (data not shown).

4. Conclusion

This study demonstrates that the prelactoproteins synthesized in an heterologous cell free system in the presence of mammary microsomal membranes are segregated in the lumen of these vesicles and accurately processed into authentic lactoproteins as revealed by partial amino terminal sequence analyses. The unexpected behavior of processed β- and α_{s1}-caseins (data not shown) in SDS-gel electrophoresis which is difficult to explain in terms of molecular weight, but might correspond to a decrease in the SDS binding or to conformational differences between pre-caseins and their authentic counterparts. An alternative which cannot be ruled out, is that processing and phosphorylation might be coupled and occur during the transfer of these proteins across the membranes as demonstrated for the glycosylation process [17,18].

Both our results and those in [14] demonstrate clearly that the enzyme(s) responsible for the cleavage of the 'signal peptide' can be delocated from the microsomal membranes by sodium deoxycholate and retains activity and specificity. The enzyme(s) isolated from mammary microsomal membranes was able to convert post-translationally >70% of β- and α_{s1}-pre-caseins into their authentic counterparts. Preliminary experiments carried out on pre-β-lactoglobulin showed that this protein appears to be a poor substrate for the solubilized signal peptidase(s) and suggested that the folding of the proteins can in some case represent a limiting factor for the post-

translational cleavage of the 'signal peptide'. In this respect, it should be noted that β-lactoglobulin exists predominantly as a dimer unit [19]. If synthesized pre-β-lactoglobulin molecules have the same propensity to associate, the signal peptide might be inaccessible to enzymatic attack. A conformational change could be induced in the signal region by the formation of a disulfide bond between the two Cys residues, thus masking the cleavage site.

The exact nature of the enzymatic activity which converts pre-proteins into their authentic counterparts remains to be elucidated. Experiments to characterize products of cleavage by gel filtration have been negative. The failure to detect such peptides does not rule out the involvement of an endopeptidase in the removal of the signal, but suggests a quick breakdown of the latter.

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