REVERSIBLE CALCIUM INHIBITION OF THE MEMBRANE-DEPENDENT CLEAVAGE OF PRE-PLACENTAL LACTOGEN IN ASCITES CELL-FREE EXTRACTS

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1. Introduction

Messenger RNAs that encode for secretory proteins direct the synthesis in various cell-free systems of precursor forms (designated pre) containing an extra N-terminal sequence of about 25 amino acids [1–8]. It has been suggested that the extra protein portion plays a role in mediating the formation of membrane-bound ribosomal complexes [9,10].

Various prepeptides have been cleaved to their corresponding finished products in mammalian cell-free extracts by an added membrane fraction derived from the endoplasmic reticulum [10–13]. Cleavage is exerted only on the growing nascent chains and released chains are refractory to cleavage [10,11] and, therefore, as observed, preproteins would not accumulate intracellularly. Thus, this cleavage reaction plays a pivotal role in the maturation of secretory proteins and represents a potential point for controlling their extracellular levels.

The processing of prepeptides is thought to involve the following sequential steps [10–12]:

(i) Synthesis of nascent chains (bearing the prepor-tion) to an appropriate length.
(ii) Binding of the ribosome-nascent chain to the membrane.
(iii) Discharge of the protein through the membrane.
(iv) Scission of the prepeptide.

One of the difficulties in attempting to dissect this reaction is that the cleavage event is coupled to translation, and that completed chains cannot serve as substrates for the cleavage process. Thus, a specific reversible inhibitor of this membrane-dependent reaction would be very useful for studying its role in the secretory pathway, especially if it did not markedly affect the rate of mRNA translation. For example, such an inhibitor would be useful for isolating various ribosomal-membrane intermediates that accumulate during the reaction, as well as providing some information regarding the nature of the protease activity. Here we show that calcium reversibly inhibits cleavage of prehPL to hPL in ascites cell-free extracts. This inhibition occurs at concentrations that do not affect the over-all rate of protein synthesis.

'Pre-protein' as used here refers to the initial in vitro translation product from mRNAs encoding secretory proteins

2. Materials and methods

Ribosomes, ribosome-free supernate (S-100) and the preincubated 30 000 × *g* post mitochondrial supernate (S-30) were prepared from Krebs ascites tumor cells [11,12]. To obtain ribosomes and S-100, the preincubated S-30 was placed on a layer of 1.0 M sucrose containing buffer A (30 mM Tris–HCl (pH 7.5), 120 mM KCl, 5 mM magnesium acetate, 7 mM 2-mercaptoethanol) and centrifuged for 5 h at 40 000 rev/min in a Spinco 60 Ti rotor.

Ascites membranes were isolated by collecting the material that accumulated at the interphase of the S-100 and the sucrose cushion, resulting from the above ribosomal centrifugation. The membranes were then diluted with 6 vol. buffer A and centrifuged at 200 000 × *g* for 2 h. The pellet was resuspended in buffer A to give a concentration for 20–40 A_{260} units/ml.

RNA-dependent protein synthesis was assayed in a 0.18 ml reaction mixture as described [12]. Approximately 150 μg S-100 protein and 5 μg ribosomes were added to each reaction mixture. Where indicated, 25 μg of membranes were added. When trypsin was used in the cell-free system, 25 μg was added to the reaction mixtures after incubating them for 60 min; this was followed by a subsequent incubation for 30 min. The products synthesized in vitro were examined on 20% polyacrylamide slab gels [11].

3. Results

In the reconstituted ascites cell-free system (S-100 and ribosomes) term placental mRNA directs the synthesis of prehPL [11]. When membranes derived from the endoplasmic reticulum of ascites tumor cells were added within the first few minutes of incubation, prehPL was converted to hPL (fig.1). The presence of 0.2 mM calcium inhibited the membrane-dependent conversion of prehPL (fig.2). This was observed when calcium was added at the start of or after 5 min of incubation. This concentration of calcium had little effect on the overall rate of protein synthesis in the ascites cell-free system (data not shown). The optimal range for inhibition was between 0.1 mM and 0.2 mM. As expected, calcium added after 60 min, at a time when processing and translation were complete [11], had no effect on the appearance of the cleaved product. The inhibition was completely reversed by the subsequent addition of EGTA (fig.3). It is important to note that EGTA was added after the system was exposed to calcium for 5 min. Thus, these experiments demonstrate that calcium reversibly inhibits the membrane-dependent conversion of prehPL to hPL.

If, as suggested [9,10], processing of prepeptide requires binding of the ribosome-nascent chain to the membrane prior to scission of the prepeptide, prehPL would be observed if calcium interfered with either the binding or the proteolytic steps. To investigate this point, the ability of reticular membranes to

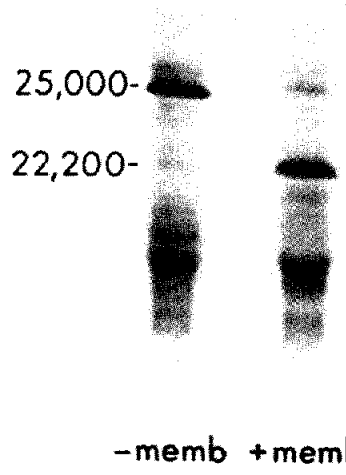

Fig.1. Sodium dodecylsulfate (SDS) gel electrophoresis of proteins synthesized in response to placental mRNA in the presence and absence of membranes. Membranes were added at the start of incubation. The amount of protein and radioactivity (100 000 cpm) in each lane was the same. The migration of hPL (mol. wt 22 200) is shown.

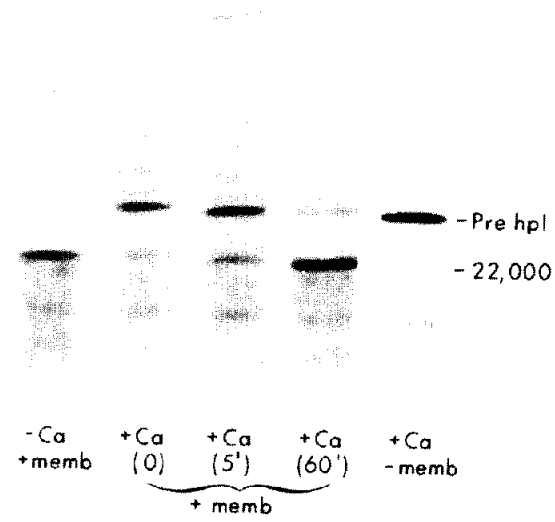

Fig.2. Sodium dodecylsulfate (SDS) gel electrophoresis of proteins synthesized in the presence of 0.2 mM calcium chloride. Membranes were added at the start of incubation. At the indicated times calcium chloride was added. The amount of radioactivity added was 100 000 cpm. The reaction mixtures were incubated for a total of 90 min.

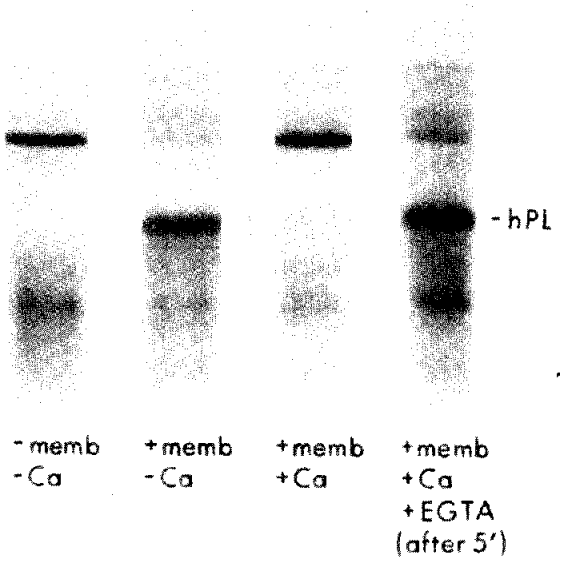

Fig.3. The effect of 2 mM EGTA on the conversion of prehPL to hPL. Where indicated, EGTA was added after 5 min incubation. Calcium and membranes were added at the start of incubation. Incubation was for 90 min.

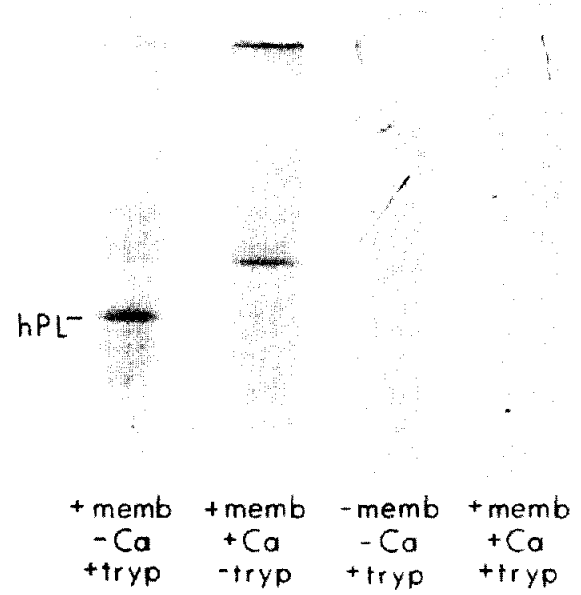

Fig.4. Effect of trypsin on prehPL synthesized in the presence of calcium. Calcium and membranes were added at the start of incubation. Where indicated, 15 μg trypsin was added after 60 min incubation. Trypsin treated reactions were then incubated for an additional 30 min. Approximately 50 000 cpm of radioactivity was added to each lane.

sequester the cleaved product and protect it against exogenously added proteolytic enzymes was employed [10]. If ribosomes bearing nascent chains bind to the membranes in the presence of calcium, it might be expected that prehPL chains would accumulate in the vesicle if the putative cleavage enzyme was inhibited. If prehPL accumulates in the vesicle it should be insensitive to trypsin digestion. However, if calcium prevented binding of the ribosome–nascent chain complex to the membrane, prehPL chains synthesized would be susceptible to proteolytic digestion.

Accordingly, reaction mixtures incubated in the presence or absence of calcium, were subjected to protease attack (fig.4). Trypsin was added after 60 min incubation and the reaction mixtures incubated an additional 30 min. In the absence of calcium, the membrane-dependent cleaved product was protected from trypsin hydrolysis, whereas, prehPL synthesized in the absence of membranes was degraded by trypsin (fig.4). In the presence of calcium, the addition of membranes at the start of the incubation did not protect prehPL from trypsin degradation.

4. Discussion

These data demonstrate that calcium specifically inhibits the membrane-dependent cleavage of prehPL to its mature hormone form. The inhibition is reversible and is complete. Similar observations were seen with similar concentrations of zinc ion (data not shown). The concentrations of these divalent ions that inhibit processing have little effect on the rate of translation of the mRNA.

Since prehPL synthesized in the presence of calcium and membranes was not protected from protease digestion, it appears that prehPL did not accumulate in the membraneous vesicles. These data suggest that calcium interferes with the presumed association step of the ribosome–nascent chain

complex to the membranes. Alternatively, penetration of the protein portion bearing the prepeptide into the membrane may require cleavage for pulling the protein into the vesicle. Therefore, the cleavage enzyme may be inhibited and prehPL would accumulate on the cytoplasmic side of the vesicle and thus be sensitive to trypsin hydrolysis.

Because calcium and zinc are capable of forming complexes with microsomal membranes [14–16], it is possible that these divalent cations are inducing the formation of membrane aggregates. Thus, the membranes might not be accessible to the nascent chains of prehPL. However, at the concentration of calcium used here no changes in the light scattering for a comparable concentration of membranes was observed. Only when the concentration of calcium exceeded 1.4 mM was there an increase in light scattering which would suggest aggregate formation. Significantly, in contrast to its effect on cleavage, EGTA did not reverse the light scattering changes observed at these higher concentrations of calcium.

Of interest, other investigators have shown that zinc ions inhibit specific proteolytic cleavages of mammalian viral proteins in their host cell [17–19]. While the mechanism is unclear it supports the data presented here that the inhibition of cleavage of prehPL to hPL is related to an alteration of the preprotein processing activity associated with membranes derived from the endoplasmic reticulum.

Acknowledgements

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