

A RECEPTOR FOR SIGNAL SEGMENTS OF SECRETORY PROTEINS IN ROUGH ENDOPLASMIC RETICULUM MEMBRANES

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1. Introduction

It is generally assumed that the cotranslational transfer of polypeptides across the ER membrane is initiated by the specific recognition of a signal peptide in the nascent secretory protein by the rough ER membrane [1,2]. We have devised a simple post-translational binding assay which demonstrated the existence of specific receptors on the cytoplasmic side of the rough ER membrane which bind the signal sequence of in vitro synthesized carp preproinsulin [3]. This binding occurred even in the absence of ribosomes in contrast to the cotranslational vectorial transport of the nascent protein.

Here, we show that the precursor to human placental lactogen is bound to rough ER membranes from dog pancreas as is carp preproinsulin. There is an effective competition between the two preproteins for the membrane receptor. It is shown that the binding sites determined post-translationally are identical with the signal peptide receptors functioning during vectorial transport of the nascent chain. The binding of signal peptides to the receptor is insensitive to 0.5 M KCl but is completely inhibited by Ca^{2+} ($K_i \approx 20 \mu\text{M}$). The latter inhibition can be released by EGTA. As a first step toward the purification of the signal peptide receptor it is shown that partial solubilization of the ER membrane by 0.2% deoxycholate does not reduce the binding activity.

This work provides evidence for the existence of a

general signal peptide receptor on rough ER membranes. The properties described here clearly distinguish the signal peptide receptor from the ribosome receptor.

2. Materials and methods

2.1. Preparation of mRNA

Poly(A)-rich RNA from carp islets (Brockmann bodies of *Cyprinus carpio*) was isolated as in [3,4]. Placental RNA was isolated from human term placenta as in [5]. It was purified by two rounds of poly(U)-Sephrose chromatography [4]. Globin mRNA was isolated from polyribosomes of rabbit reticulocytes and was a kind gift of B. Thiele (Institut für Physiologische und Biologische Chemie, Berlin).

2.2. Cell-free synthesis

The conditions for cell-free synthesis in a wheat germ system were essentially those in [6] with minor modifications [3]. The assay contained in 50 μl final vol.: 20 μl 30 000 \times g supernatant (containing 1.4–1.6 A_{260} -units), 5 μCi [^3H]leucine (50 Ci/mmol, The Radiochemical Centre Amersham) and 0.3–0.8 μg mRNA. The final concentrations of other substances were: Tris-HCl (pH 7.7), 19 mM; KCl, 70 mM; magnesium acetate, 3.5 mM; dithioerythritol, 2.5 mM; creatine phosphate, 8 mM; creatine kinase, 74 $\mu\text{g}/\text{ml}$; ATP, 1.2 mM; GTP, 0.25 mM and amino acids (except leucine) 20 μM . Incubation was for 60 min at 25°C.

2.3. Product analysis

This was done by SDS-polyacrylamide gel electrophoresis according to Laemmli [7]. A 12–22% linear

Abbreviations: ER, endoplasmic reticulum; EGTA, ethylene glycol-bis-(2-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS, sodium dodecylsulfate

acrylamide gradient gel was used with a 5% stacking gel. The gel was processed for fluorography as in [8] and exposed to a preflashed X-ray film (Kodak, Royal X-Omat) at -70°C .

2.4. Preparation of membranes

Rough and smooth ER membranes were isolated from dog pancreas as in [9] with minor modifications [3]. Rough ER membranes were stripped of ribosomes by treatment with EDTA [3,9]. Erythrocyte plasma membranes were prepared from rabbit erythrocytes by osmotic lysis of the cells and repeated washing of the $10\,000\times g$ pellet. All membranes were dialyzed before use for 1 h against buffer A (10 mM Tris-HCl (pH 7.7), 74 mM KCl; 3.5 mM magnesium acetate; 3.5 mM 2-mercaptoethanol).

2.5. Binding assay

The assay was performed as in [3]. It contained in 50 μl final vol.: 20 μl 30 000 $\times g$ supernatant from wheat germ containing [^3H]leucine-labelled cell-free translation products and up to 16 μl membrane suspension (corresponding to 20–70 μg membrane protein). The assay was performed in buffer A. After incubation at 25°C for 15 min the samples were cooled to 0°C and centrifuged for 60 min in a microfuge. The membrane pellet was washed twice with 300 μl buffer B (same as buffer A except that KCl was 115 mM) followed each time by a 1 h centrifugation in the microfuge. The final pellet was dissolved in 20 μl 0.1 M NaOH. After addition of 15 μl 1 M Tris-HCl (pH 7.6) and 10 μl 10% human serum albumin the samples were precipitated with 10 ml 5% trichloroacetic acid. After incubation at 100°C the precipitates were collected on Whatman GF/A filters and counted. The combined supernatant and washing solutions were also precipitated with trichloroacetic acid and processed for counting.

3. Results and discussion

3.1. Binding of preproinsulin and prelactogen to the signal receptor

In order to test the binding of signal peptides to rough ER membranes a simple assay has been devised [3]. Poly(A)-rich RNA from either carp islets or human placenta was translated in a wheat germ cell-free protein synthesizing system in the presence of [^3H]leucine. The predominant translation products

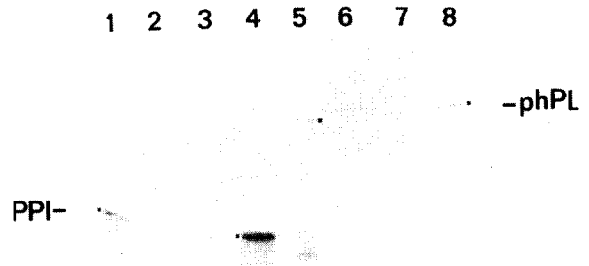


Fig.1. Specific binding of preproinsulin and prelactogen to rough ER membranes. Cell-free translation of poly(A)-containing RNA from carp islets or from human placenta was carried out in a wheat germ system. The labelled translation products were added to either rough (24 μg protein) or smooth (20 μg protein) ER membranes and after incubation the membranes were sedimented to determine the bound and unbound fractions as in section 2. After precipitation with trichloroacetic acid, both the membrane and supernatant fraction were taken up in SDS sample buffer and applied to SDS-polyacrylamide gel electrophoresis. Lanes (1–4), samples incubated with cell-free products coded by carp islet mRNA; lanes (5–8), samples incubated with cell-free products coded by human placenta mRNA; lanes (1,5), material not bound to smooth membranes; lanes (2,6), sedimented smooth membranes; lanes (3,7), supernatants after incubation with rough membranes; lanes (4,8), material bound to rough membranes. PPI and phPL denote the positions of preproinsulin and prelactogen, respectively. These bands are also indicated by dots.

were preproinsulin and prelactogen, respectively (fig.1, lanes 1,5). The labelled reaction products were added to rough ER-membranes from dog pancreas which were stripped of ribosomes by EDTA-treatment. After incubation the membranes were sedimented by centrifugation, washed twice and the acid-precipitable radioactivity bound to the membranes determined.

As shown in table 1, a high percentage of the cell-free products coded by carp islet or human placenta mRNA was bound to the rough ER membranes. On the other hand, there was much less binding to smooth membranes isolated from dog pancreas. The specificity of the binding reaction was demonstrated by the fact that proteins lacking signal sequences, such as rabbit globin (table 1) or carp proinsulin [3], were not bound. Furthermore, binding was not affected by addition of excess of insulin [3]. The translation products coded by mRNA from carp islets and from human placenta competed strongly with each other for the binding sites on the rough ER membranes (table 1). The fact

Table 1
Competitive binding to rough endoplasmic membranes of cell-free translation products coded by carp islet and human placenta mRNAs

Endoplasmic membranes	Cell-free products added to the membranes during preincubation	Cell-free products added to the assay after preincubation	Radioactivity bound to the membranes (%)
Rough	—	[³ H]Preproinsulin	63.4
Smooth	—	[³ H]Preproinsulin	14.0
Rough	[¹ H]Prelactogen	[³ H]Preproinsulin	11.7
Rough	[³ H]Preproinsulin	[¹ H]Prelactogen	21.1
Rough	[¹ H]Endogenous	[³ H]Preproinsulin	70.2
Rough	[³ H]Preproinsulin	[¹ H]Endogenous	59.6
Rough	—	[³ H]Prelactogen	78.3
Smooth	—	[³ H]Prelactogen	14.5
Rough	[¹ H]Preproinsulin	[³ H]Prelactogen	20.1
Rough	[³ H]Prelactogen	[¹ H]Preproinsulin	28.4
Rough	[¹ H]Endogenous	[³ H]Prelactogen	77.5
Rough	[³ H]Prelactogen	[¹ H]Endogenous	80.1
Rough	—	[³ H]Globin	16.2

Cell-free translation products were synthesized in a wheat germ system using carp islet or human placenta mRNA. The products are designated 'preproinsulin' and 'prelactogen', respectively. Products obtained in the absence of added mRNA are called 'endogenous'. The cell-free products were either labelled with [³H]leucine ([³H]preproinsulin, [³H]prelactogen) or unlabelled ([¹H]preproinsulin, [¹H]prelactogen, [¹H]endogenous). The unlabelled proteins used for the competition studies were added in a 6-fold excess over the labelled species. The competitor was added to the membranes either prior to the labelled proteins during a preincubation or after the labelled proteins had been bound. Both preincubation and the second incubation were for 15 min at 25°C. Separation of the bound and nonbound material was carried out as in section 2. Cell-free synthesized rabbit globin served as a control in one experiment. In each assay 12 µg rough or 10 µg smooth endoplasmic membranes were used (values are given as membrane protein)

that nearly the same values were obtained when unlabelled competitor was added before or after binding of the labelled species indicates that binding equilibrium was obtained within 15 min.

Earlier competition experiments [19,20] carried out in a cotranslationally processing system did not permit a specification of the site of competition which was considerably less than reported here.

To demonstrate that the sites involved in post-translational binding are also those which function as signal receptors during the vectorial transport of nascent proteins, membranes preincubated with completed, unlabelled preproteins were used in a cotranslational processing assay with the corresponding mRNA. It may be seen (fig.1) that the processing was greatly inhibited indicating that the completed preproteins

had blocked the signal receptor. In other experiments (not shown) there was also blocking of the processing by the unrelated preprotein, again indicating a common receptor. Membranes not preincubated with preproteins carried out processing (fig.1) and this was accompanied by vectorial transport across the membrane as shown by the inaccessibility of the labelled proteins to added proteases ([3], unpublished). There was no inhibition of the cotranslational processing if endogenous wheat germ cell-free products or cell-free synthesized rabbit globin were used for the preincubation of the membranes (fig.1). These controls demonstrate that the inhibition of processing was not due to the effect of other components of the translation mixture which were added together with the preproteins.

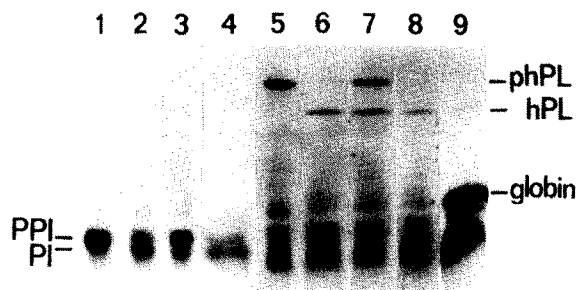


Fig.2. Inhibition of cotranslational processing of preproteins by presaturation of signal receptors in rough ER membranes. Rough ER membranes from dog pancreas (12 μ g protein) were preincubated for 15 min at 25°C with unlabelled cell-free translation products coded by carp islet or human placenta mRNA (8 μ l original cell-free assay). The preincubated membranes were then used in a cell-free assay programmed with either carp islet or human placenta mRNA. The [³H]leucine-labelled products were analyzed on a SDS-polycrylamide gel (~10 000 cpm/gel slot). Controls were carried out with endogenous wheat germ translation products or with in vitro synthesized rabbit globin, both labelled with [³H]leucine. Lanes (1–4), cell-free products coded by carp islet mRNA; lanes (5–9), cell-free products coded by human placenta mRNA; lanes (1,5), without addition of membranes; all other lanes received samples incubated with membranes: lanes (2,6), untreated membranes; lanes (3,7), membranes preincubated with unlabelled cell-free products coded by carp islet and human placenta mRNA, respectively; lanes (4,8), membranes preincubated with endogenous wheat germ products; lane (9), membranes preincubated with labelled rabbit globin. PPI, PI, phPL and hPL denote the positions of preproinsulin, proinsulin, prelactogen and lactogen, respectively.

Fig.2 shows that preproinsulin and prelactogen were the major translation products bound post-translationally to rough ER membranes. In agreement with the data in table 1, both preproteins were not bound to smooth membranes.

These data provide evidence that the binding of signal peptides to rough ER membranes is not a unique property of carp preproinsulin but is also observed with human placental prelactogen, another, unrelated secretory protein containing a signal sequence at its N-terminus [10]. Moreover, the competition experiments show that the completed preproteins are recognized by the same receptor. Since binding requires the interaction with the signal segment, it can also be concluded that it remains exposed after the polypeptides are completed and released from the ribosomes.

3.2. Salt dependence of the binding of signal peptides

It is known that the binding of ribosomes to rough ER membranes, observed in the absence of mRNA and nascent protein chains, is salt sensitive [11,12]. Since binding of ribosomes and of signal peptides to rough ER membranes are functionally related processes, it was of interest to determine the effect of high salt concentrations on the binding of signal peptides. The results in table 2 demonstrate, that the specific binding of signal peptides to rough ER membranes was unaffected by incubation with 0.5 M KCl. Increasing concentrations of KCl appeared to decrease only the unspecific background binding as shown also by the decrease in 'binding' of rabbit globin (table 2). The percentage of specific binding measured as the difference between total binding and globin binding remained almost constant.

The data in table 2 are in clear contrast to the salt sensitivity of ribosome binding [11,12]. These results and the lack of competition between signal peptides and ribosomes [3] demonstrate that the two receptors reside in different sites. Furthermore, the data suggest that electrostatic interactions may be of importance for ribosome binding but not for binding of the signal peptide.

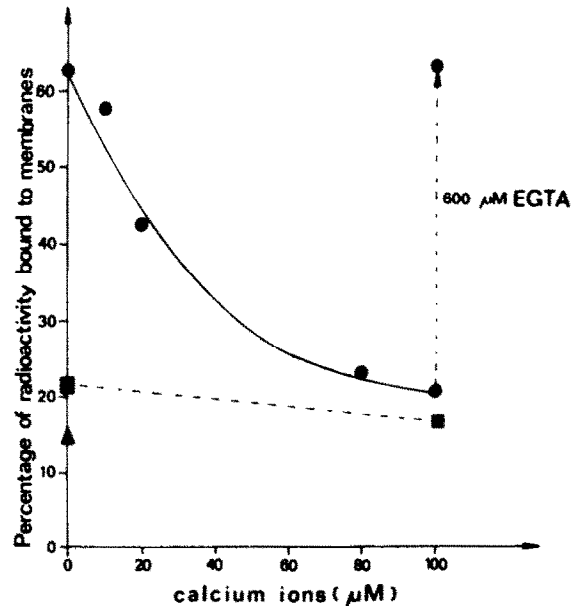
Our results agree with [13] where rough ER membranes washed with 0.5 M KCl were found to carry out cotranslational processing of preproteins.

Table 2
The influence of high salt concentrations on the binding of cell-free products to rough endoplasmic membranes

Labelled cell-free products coded by	[KCl] (M)	Radioactivity bound to the membranes (%)
Carp islet mRNA	0.074	66.2
	0.3	57.7
	0.5	48.3
Rabbit globin mRNA	0.074	22.9
	0.3	12.6
	0.5	6.7

Cell-free translation was carried out in a wheat germ system using carp islet poly(A)-containing RNA or globin mRNA from rabbit reticulocytes. The binding of the labelled translation products to rough endoplasmic membranes from dog pancreas (19 μ g membrane protein) was tested in the presence of different concentrations of KCl as in section 2. The washing steps were carried out with buffer containing the same [KCl] as present during the binding reaction

Fig.3. Inhibition of binding of preproinsulin to the signal receptor by Ca^{2+} . Membranes were preincubated with $CaCl_2$ for 15 min at 25°C. A further incubation for 15 min at 25°C was carried out after addition of labelled translation products synthesized in a wheat germ system programmed with carp islet mRNA (10 μ l cell-free products added to 40 μ l preincubation mixture). The final $[Ca^{2+}]$ is given in the figure. The % of radioactivity bound to the membranes was determined as in section 2. (●) Binding to rough ER membranes (19 μ g membrane protein); (■) binding to smooth ER membranes (13 μ g membrane protein); (▲) binding of cell-free synthesized rabbit globin to rough ER membranes.



3.3. The effect of Ca^{2+} on the binding of signal peptides

Inhibitors of the vectorial transport or of the processing of secretory proteins have been searched for with limited success [14–16]. Ca^{2+} has been reported to inhibit either the vectorial transport itself or the prior binding of the signal peptide [17]. We have therefore tested the effect of Ca^{2+} in our post-translational binding assay which allows us to separate binding from the transport process. As shown in fig.3, Ca^{2+} inhibited completely the binding of preproinsulin to rough ER membranes and this inhibition was re-

leased by addition of EGTA. A K_i -value of $\sim 20 \mu$ M was calculated from the data of two different experiments. This value fits with that determined for the overall effect of Ca^{2+} on the processing of placental prelactogen [17].

Although the mechanism of the Ca^{2+} effect is yet

Table 3
The effect of deoxycholate on the binding of cell-free translation products to rough ER membranes

Membranes	Additions for preincubation	Additions to the assay after preincubation	Sedimentation procedure	Radioactivity bound to the-membranes (%)
REM	[3 H]Preproinsulin	—	1 h, 10 000 \times g	41.1
EPM	[3 H]Preproinsulin	—	1 h, 10 000 \times g	3.6
REM	[3 H]Globin	—	1 h, 10 000 \times g	12.1
EPM	[3 H]Globin	—	1 h, 10 000 \times g	12.5
REM	[3 H]Preproinsulin	—	16 h, 90 000 \times g	41.3
REM	Deoxycholate	[3 H]Preproinsulin	16 h, 90 000 \times g	44.2
REM	[3 H]Preproinsulin	Deoxycholate	16 h, 90 000 \times g	37.5
REM	[3 H]Globin	Deoxycholate	16 h, 90 000 \times g	8.8
EPM	Deoxycholate	[3 H]Preproinsulin	16 h, 90 000 \times g	12.0
EPM	[3 H]Preproinsulin	Deoxycholate	16 h, 90 000 \times g	15.7

Cell-free translation was carried out in a wheat germ system using mRNA from carp islets or from rabbit reticulocytes. The products were labelled with [3 H]leucine and are designated as [3 H]preproinsulin and [3 H]globin, respectively. Binding of the labelled products to rough ER membranes from dog pancreas (REM) (19 μ g membrane protein) or to erythrocyte plasma membranes (EPM) (70 μ g membrane protein) was determined either by the procedure in section 2 (centrifugation for 1 h at $\sim 10\,000 \times g$) or by sedimentation for 16 h at $90\,000 \times g$ in a Spinco Ti 60 rotor (Beckmann L2 centrifuge). Deoxycholate (final conc. 0.2% (w/v)) was added to the membranes either before or after binding of the cell-free translation products. Incubation with detergent was for 30 min at 25°C, with cell-free products for 15 min at 25°C

obscure it may be assumed that Ca^{2+} affects the receptor in an allosteric manner. This effect may be similar to that of Ca^{2+} on contractile proteins. Although Ca^{2+} in other systems has been shown to affect phosphorylation processes, it should be noted, that the binding itself is independent of ATP (unpublished).

3.4. Subfractionation of membranes containing signal receptors

A purification of the signal peptide receptor will require its separation from other membrane proteins with retention of the signal binding activity. We therefore tested the effect of concentrations of deoxycholate which disrupt the membrane into smaller fragments and lead to the release of luminal content proteins and to partial solubilization of membrane proteins [18]. Membrane remnants could still be sedimented using a slightly modified version of our usual binding protocol (table 3). As seen from table 3, 0.2% deoxycholate did not release the bound preproinsulin from the receptor nor did it prevent the binding if added prior to preproinsulin. Thus, deoxycholate at this concentration does not disturb the functional integrity of the signal receptor and may be a valuable tool for purification.

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