gene expression and regulation:

the legacy of LUIGI GORINI

Proceedings of the Symposium, Gene '87, held in Milan, Italy, 12–14 October 1987

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1988

EXCERPTA MEDICA, Amsterdam — New York — Oxford

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International Congress Series No. 784
ISBN 0 444 80993 7

Published by:
Elsevier Science Publishers B.V.
(Biomedical Division)
P.O. Box 211
1000 AE Amsterdam
The Netherlands

Sole distributors for the USA and Canada:
Elsevier Science Publishing Company Inc.
52 Vanderbilt Avenue
New York, NY 10017
USA

Library of Congress Cataloging in Publication Data:

Gene expression and regulation : the legacy of Luigi Gorini ;
proceedings of the symposium held in Milan, Italy, 12-14 October
1987 / editors, Mina Bissell ... [et al.].
p. cm. -- (International congress series ; no. 784)
Includes bibliographies and index.
ISBN 0-444-80993-7 (U.S.)
1. Gene expression--Congresses. 2. Genetic regulation-
-Congresses. 3. Gorini, Luigi, 1903-1976. I. Gorini, Luigi,
1903-1976. II. Bissell, Mina. III. Series.
[DNLM: 1. Gene Expression Regulation--congresses. W1 EX89 no.
784 / QH 450 G3263 1987]
QH450.G4618 1988
574.87'322--dc19
DNLM/DLC
for Library of Congress 88-16507
CIP

Bayerische Staatsbibliothek München

Printed in The Netherlands

Cover illustration by Pietro Alano

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Bissell, Deho, Sironi and Torriani, editors

IMPORT OF HONEYBEE PREPROMELITTIN INTO THE ENDOPLASMIC RETICULUM

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INTRODUCTION

Every polypeptide has a specific function as well as a unique functional location, i.e. an intra- or extracellular location where it fulfills its function (1,2). There are two facts which turned the latter into a central problem in our understanding of gene expression in the eukaryotic cell: i) Neglecting mitochondria and chloroplasts, there is only one site of protein synthesis, the cytoplasm, but there are many different potential functional locations, including the cell organelles and the extracellular space. ii) The site of synthesis is separated from these locations by phospholipid bilayers. Therefore, there must exist mechanisms which guarantee the specific transport of proteins across membranes and the assembly of proteins into membranes, respectively.

We are employing honeybee prepromelittin (70 amino acid residues), the precursor of the secretory protein promelittin (49 amino acid residues), as a tool for elucidating the mechanism of import of proteins into the endoplasmic reticulum (3-8). The logic behind using this small precursor protein is that it has a typical signal sequence (21 amino acid residues), is prcocessed by signal peptidase and thus presumably shares some steps of the import pathway with larger precursor proteins, and that it may be able to bypass certain steps which are obligatory for larger precursors, possibly because of a lower tendency for folding into structures that are incompatible with membrane insertion or transport. An additional reason for choosing this small precursor protein is that it can be expected to be imported posttranslationally because of its size and, therefore, to be a technical advantage. Since approximately 40 amino acid residues are shielded within the ribosome, there is not much time between the point where a 21 residue long signal peptide emerges from the ribosome, and becomes free to interact with a receptor, and the point of termination of protein synthesis.

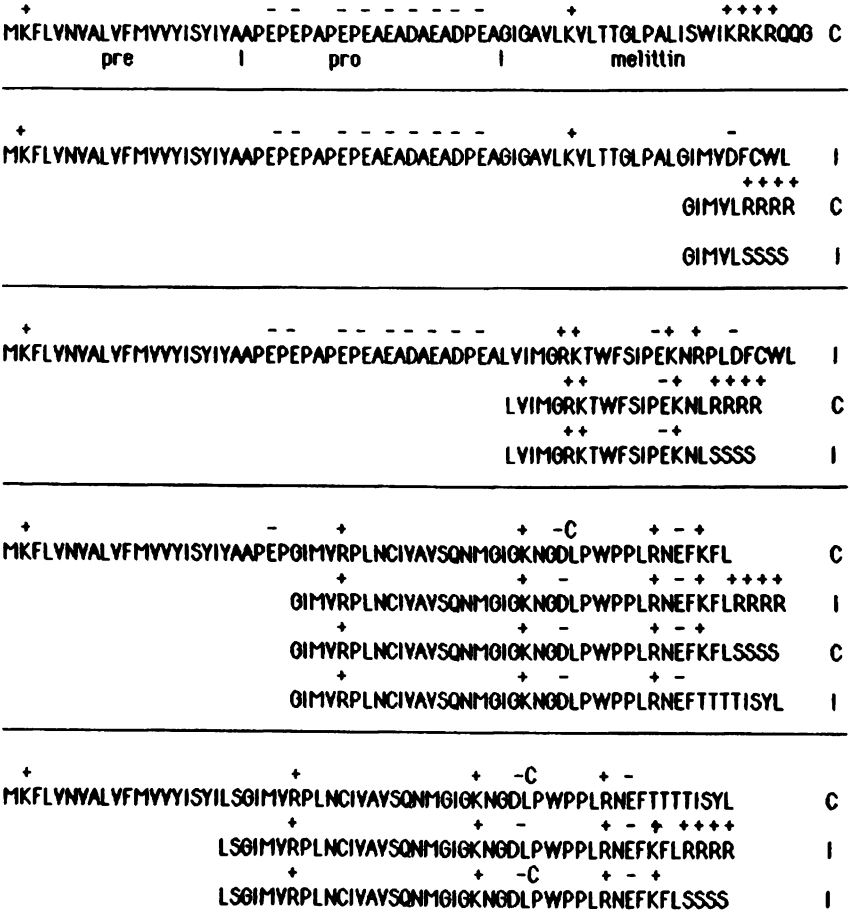

Figure 1. Amino acid sequence of prepromelittin and related precursor proteins, derived from hybrid proteins between prepromelittin and dihydrofolate reductase. Four classes of small precursor proteins related to prepromelittin are shown (for terminology refer to: 4). The different classes of precursors are identical with prepromelittin to varying degrees (indicated for one protein within a class). The different precursors within a class are different from each other within the region which is not related to prepromelittin (indicated for all proteins within a class). The precursors are termed competent (C) or incompetent (I) with respect to import into microsomes (4). Amino acid sequences are given in single letter code. Charged amino acid side chains and the boundaries between the pre, pro, and mature regions are indicated. Three precursor proteins were subjected to oligonucleotide directed mutagenesis which gave rise to three derivatives, containing a single substitution (leucine to cysteine) (for terminology see: 5). This is indicated by superscribed C.

MATERIALS AND METHODS

Our experimental system involves plasmids, containing the cDNAs of interest and suitable for transcription in vitro, rabbit reticulocyte lysates and dog pancreas microsomes (3-12). We assay association of precursor proteins with microsomes in binding experiments where precursors are incubated with microsomes at low temperature and microsomes are reisolated by gradient centrifugation (5). Membrane insertion is assayed as removal of the signal peptide by signal peptidase on the luminal side of the membrane (3). The assays for complete translocation involve testing sequestration, i.e. protection against externally-added protease in the absence of detergent but sensitivity in the presence of detergent (3,4), and localization, i.e. subfractionation of microsomes at neutral and alkaline pH-values (4).

RESULTS

Competence of Prepromelittin for Import into Microsomes

Prepromelittin is correctly processed and imported by dog pancreas microsomes (3). Import of prepromelittin is not dependent on signal recognition particle (SRP) and docking protein (3,7) and, as concluded by analogy with frog prepropeptide GLa and M13 procoat protein (9-11), ribosomes and a ribosome receptor (see below). The primary structure of prepromelittin, however, is crucial for import competence (4): Hybrid proteins between prepromelittin, or carboxy-terminally truncated derivatives, and the cytoplasmic protein dihydrofolate reductase from mouse were constructed. These hybrid proteins were analyzed for membrane insertion and sequestration into microsomes. The results suggest the following: i) The signal sequence of prepromelittin is capable of interacting with the SRP/docking protein-system, but this interaction is not mandatory for import of small precursor proteins (<80 amino acid residues) (Figure 1). This view is supported by the facts that the interaction between a signal peptide and SRP requires the nascent chain to be bound to a functional ribosome (13) and that the small precursor proteins should be completed and released from the ribosome by the time an interaction with SRP could occur (see INTRODUCTION). ii) In prepromelittin and related small precursor proteins a single (or a cluster of) negatively charged amino acid(s) near the amino terminus of the mature part must be balanced by a single (or a cluster of) positively charged amino acid(s) near the carboxy terminus (or charged amino acid residues must be absent from both these positions altogether) in order to create a precursor protein competent for import (Figure 1). Furthermore, in most cases the presence of ATP and, whenever the

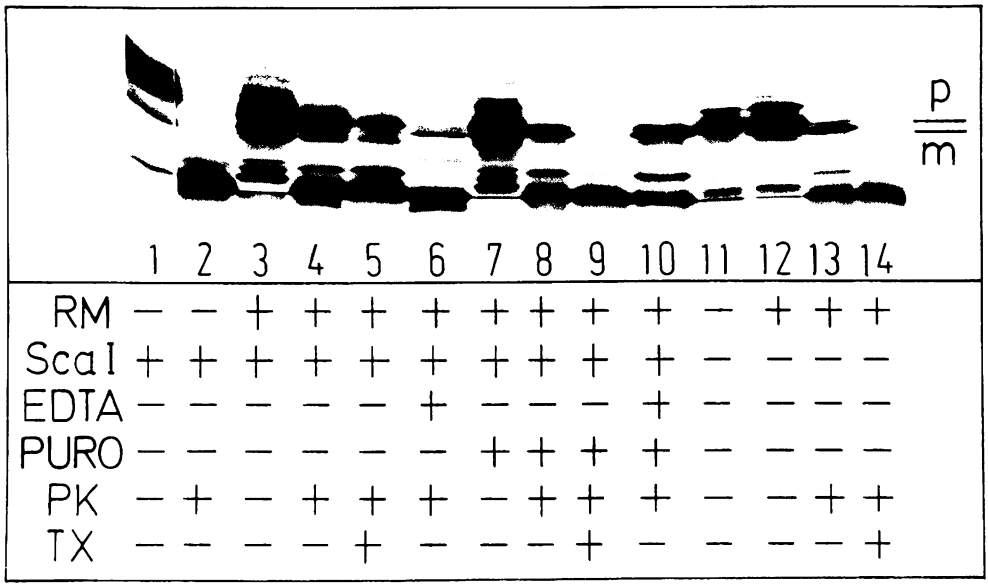

Figure 2. Posttranslational import of precursor proteins containing more than 80 amino acid residues into microsomes.

The plasmid coding for hybrid protein ppm$_{\Delta}$-DHFR/1 (4) was linearized within the coding region by the restriction enzyme Sca I and was transcribed in vitro, resulting in an mRNA without stop codon. When this transcript was used to program a rabbit reticulocyte lysate in the presence of radioactive methionine (30 min at 37°C), the major translation product was a peptidyl-tRNA (lane 1) which contained the same 94 amino acid residues as precursor ppm$_{\Delta}$-DHFR/2 (4) (lane 11). When the peptidyl-tRNA was synthesized in the presence of microsomes, little mature protein was produced and was apparently due to spontaneous release of the polypeptide from the tRNA (lane 3). This mature protein behaved like the mature protein produced during the synthesis of precursor ppm$_{\Delta}$-DHFR/2 (lanes 12-14) as it was protected against externally-added protease in the absence of detergent (lane 4) and in the presence of EDTA (5 mM) (lane 6), but was sensitive in the presence of detergent (lane 5). In addition to the mature protein, part of the precursor was protease-resistant in the absence and presence of detergent (lanes 4 and 5), but sensitive in the presence of EDTA (lane 6). Because no such precursor was observed after synthesis in the absence of microsomes (lane 2), we conclude that the protease-resistant precursor corresponds to the peptidyl-tRNA which was still hidden in the ribosome. This view is supported by the fact that the protected precursor disappeared during a subsequent incubation in the presence of puromycin (2.5 mM) and resulted in an increase of mature protein (7), i.e. gave rise to a peptidyl-puromycin which was protease-resistant in the absence of detergent (lane 8) and in the presence of EDTA (lane 10), but sensitive in the presence of detergent (lane 9).

respective precursor contains two cysteine residues, reducing conditions are a prerequisite for import (5).

Posttranslational Import of Prepromelittin into Microsomes

Import of prepromelittin is not coupled to translation (3). Furthermore, import of unrelated (9-11) and related (Figure 1) (5) precursor proteins of less than 80 amino acid residues occurs in the absence of protein synthesis and ribosomes (see above). So far, however, we have not succeeded in finding conditions for the posttranslational import of related precursor proteins (5) having more than 80 amino acids other than by artificially keeping the precursor proteins in the form of peptidyl-tRNAs associated with ribosomes (also see: 14). Such precursor proteins can associate with microsomes, through the action of SRP and docking protein, and can subsequently be chased to the mature and imported form by treatment with puromycin (Figure 2). The latter result suggests that the ribosomes, probably in collaboration with the ribosome receptor, have a function in the import of large precursor proteins beyond their role in protein synthesis. Strikingly, the same precursor proteins can be exported in E.coli, without any "tricks", in the absence of protein synthesis (8).

Competence of Prepromelittin for Association with, Insertion into and Translocation across the Microsomal Membrane

The import pathway of prepromelittin-related precursor proteins was resolved into three sequential steps (3-5): i) Binding of precursors to microsomes; ii) insertion of precursors into the membrane; and iii) complete transfer of the mature proteins across the membrane:

Association and Insertion. Binding of precursor proteins involves the counter-balancing of charged amino acid residues within the mature part, i.e. competence for import is identical with competence for binding (5). Formation of an intramolecular disulfide bridge within the mature part of competent precursor proteins containing two cysteine residues allowed association of the oxidized precursors with the microsomal membrane but reversibly inhibited their membrane insertion (5). Furthermore, formation of an intramolecular disulfide bridge within an incompetent precursor did not result in binding (Table 1), which means that the compensation of charges is the crucial feature of the competent precursor. There was no effect of ATP depletion on binding. Membrane insertion, however, was reversibly inhibited by ATP depletion (5). The effect of ATP was linked to a component of the rabbit reticulocyte lysate. Different prepromelittin derivatives were found to depend on ATP and the reticulocyte component to varying degrees (5). We conclude that binding of prepromelittin-derived precursor proteins to microsomal membranes involves a competent conformation which

TABLE I
ASSOCIATION OF PRECURSOR PROTEINS WITH MICROSOMES AT LOW TEMPERATURE

Precursor proteins were synthesized in rabbit reticulocyte lysates for 10 min at 37°C. After incubation in the presence of cycloheximide (100 µg/ml) and RNase A (80 ug/ml) for 5 min at 37°C, the samples were divided into four aliquots. Two aliquots were supplemented with water (reducing conditions), the other two aliquots with $K_3Fe(CN)_6$ (10 mM) (oxidizing conditions). One aliquot of each set of two was incubated with water (-RM), the other aliquot with microsomes (+RM) for 5 min at 4°C. The samples were divided; one part was diluted with an equal volume of double strength sample buffer, the other part was layered onto a two step sucrose gradient, subjected to centrifugation in a Beckman Airfuge (5 min at 25 psi, rotor A-100/30) and subsequently fractionated as described (5). The supernatants and the interfaces were diluted with an equal volume of double strength sample buffer and the pellets were resuspended in sample buffer. All samples were analyzed by gel electrophoresis and fluorography. Densitometric analysis of the resulting X-ray films was carried out on a LKB densitometer and the amount of precursor protein, recovered in the various fractions, was calculated as a percentage of the total precursor protein used in the assay. The existence of intramolecular disulfide bridges in the competent precursor p_{Δ}-DHFR/3Thr^C and the incompetent precursor p_{Δ}-DHFR/3Ser^C under oxidizing conditions was proved by gel electrophoresis in the absence of reducing agents (refer to 5).

	associated precursor (% of total precursor)							
	p_{Δ}-DHFR/3Thr^C				p_{Δ}-DHFR/3Ser^C			
conditions	reducing		oxidizing		reducing		oxidizing	
RM	-	+	-	+	-	+	-	+
supernatant	85.1	62.7	72.3	49.1	79.4	72.5	68.8	59.1
interface	5.4	19.8	3.3	12.9	4.2	5.7	7.2	4.9
pellet	1.7	2.9	5.1	4.2	3.5	2.6	7.9	6.7
sum	92.2	85.4	80.7	66.2	87.1	80.7	83.9	70.7

guarantees compensation of charged residues within the mature part of the precursor proteins and that this conformation has to be altered in order to allow membrane insertion to occur. In general, this conformational change is accomplished with the help of both a cytoplasmic component and ATP.

Translocation. A previously uncharacterized proteinaceous component of the microsomal membrane is required for completion of membrane transfer of promelittin and promelittin-related proteins which contain the hydrophobic part of the melittin domain (3,4).

Competence of Prepromelittin for Insertion into Detergent Micelles

On the basis of the fact that insertion of M13 procoat protein into detergent micelles is very similar to or possibly identical with membrane insertion, the effects of ATP depletion and the presence of intramolecular disulfide bridges in small precursor proteins related to prepromelittin was assayed with respect to processing by purified E.coli leader peptidase in detergent (6). Prepromelittin is correctly processed by purified leader peptidase in detergent (8). Processing in the presence of detergent showed the same prerequsites (compensation of charged amino acid residues, ATP dependence, reducing conditions in the case of precursors with two cysteine residues) as processing by microsomes. We conclude that precursors of eukaryotic secretory proteins behave similarly in this respect as compared to M13 procoat protein .

DISCUSSION

We propose the following mechanism for the import of prepromelittin into the endoplasmic reticulum (Figure 3):

A) Prepromelittin is released from the ribosome before an interaction between either the signal peptide and SRP or the ribosome and the ribosome receptor can occur. Its primary structure, however, has evolved in a way which allows it to stay competent for membrane association and insertion without the aid of these components. Possibly, a loop is formed within the mature part, thereby bringing the amino- and carboxy-terminal parts in close proximity of each other. Ionic interactions may stabilize this structure, whereas the presence of unbalanced charges in these positions may destabilize it.

B) This structure can associate with the microsomal membrane only if there is a compensation of charged amino acid residues (or absence of charged amino acid residues) at the termini of the mature part and if the signal peptide is exposed. Even disulfide bridges are allowed at this stage, but they probably never occur in vivo. It is presently unclear whether a signal

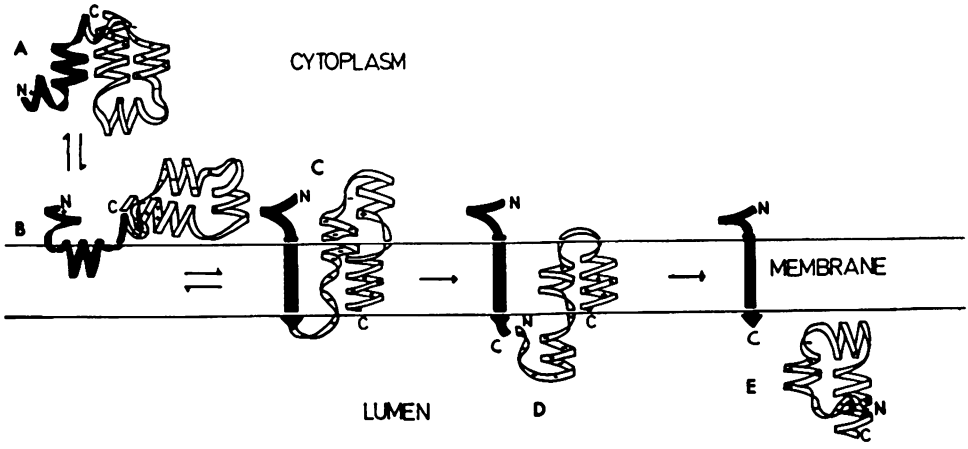

Figure 3. Working model for the import of prepromelittin and related precursor proteins into microsomes. The import of prepromelittin into microsomes occurs as a sequence of the following steps: i) Specific association of prepromelittin with the membrane (B), ii) membrane insertion of prepromelittin (C), removal of the signal peptide by signal peptidase on the luminal side of the membrane (D), and iii) complete translocation of promelittin across the membrane (E). Association involves a competent conformation and a signal peptide; a possible involvement of a signal sequence receptor (SSR) is currently under investigation. Insertion involves the signal peptide and a conformational change which typically is mediated by a soluble cytoplasmic component and the hydrolysis of ATP. Processing involves signal peptidase on the luminal side of the membrane. Translocation involves proteinaceous membrane components, at least in certain cases. Form B can be trapped in binding experiments at low temperatures, in the absence of ATP or in the presence of oxidizing agents in the case of precursors with two cysteine residues (5). Form D was observed in pretrypsinized microsomes after incubation with prepromelittin or related precursors which contain the hydrophobic melittin domain (3,4). The membrane association and insertion steps are likely to be reversible, the steps following processing are presumably irreversible. The proposed conformations are based on standard prediction methods but are, nevertheless, purely hypothetical. The signal peptide is shown in its two most probable conformations (indicated in black).

sequence receptor (SSR) (15) is involved at this step or whether it is a pure protein/lipid interaction. Strikingly, prepromelittin export in E.coli does not involve the secY protein (8).

C and D) We assume that an unfolding, i.e. destabilization of this structure, has to precede membrane insertion. Typically, this is catalzed by a cytoplasmic protein which depends on the hydrolysis of ATP for its action (also see: 11,12). Strikingly, the protein without any charged amino acid residues at the mature termini does not depend on this activity. Disulfide bridges cannot be dissociated by the action of the ATP-dependent protein. Insertion may occur in the form of a hairpin structure between the signal peptide and the mature amino terminus. This event leads to exposure of the cleavage site to the luminal side of the membrane and results in processing by signal peptidase. We assume that this takes place in a lipid environment because we observed the same characteristics for insertion into detergent micelles (also see: 11).

E) The subsequent completion of translocation of the mature protein may occur in one or more steps and may or may not involve membrane components. So far we have defined one protease-sensitive component which seems to be necessary in order to expel the hydrophobic melittin domain within promelittin out of the membrane. Accordingly, proteins which do not contain this domain do not depend on this component (also see: 9). The alternatives for the action of this component are still the ones proposed by either the signal hypothesis or the membrane triggered folding hypothesis or something in between. There may be transient proteinaceous pores involved or just the phospholipids, possibly in the form of a transient bilayer distortion, or there may be membrane proteins which do not form a pore but trigger and/or control the bilayer distortion.

While the import characteristics of small precursors appear to be exceptional in the cells of higher eukaryotic organisms, they seem to be shared by a number of proteins in lower eukaryotic cells (15-20): Import of prepro-α-factor (165 amino acid residues) into microsomes in a cell free system derived from Sacccharomyces cerivisiae also occurs posttranslationally (15-20) and does not involve ribosomes (16). Furthermore, it also depends on the primary structure of the precursor protein (18) and a cytoplasmic component which involves the hydolysis of ATP in its action (15-20).

Therefore, it seems that different strategies have evolved in order to keep precursor proteins competent for import into microsomes which depend upon both the nature of the respective precursor protein and the particular cell type.

ACKNOWLEDGEMENTS

We are thankful to Dr Günter Kreil for mRNA and cDNA coding for prepromelittin, to Drs Hermann Bujard and Dietrich Stueber for cDNA coding for dihydrofolate reductase, and Dr William Wickner for a gift of E.coli leader peptidase. Furthermore, we are grateful to Maria Sagstetter and Birgitta Kaßeckert for excellent technical assistance, to Dr Donald W. Nicholson for critical comments on the manuscript, and to Hans Wiech for artfully drawing our working model. This work was supported by grants Zi234/2-1 and Zi234/2-2 from the 'Deutsche Forschungsgemeinschaft', by grant B10 from the 'Sonderforschungsbereich 184: Molekulare Grundlagen der Biogenese von Zellorganellen', and by the 'Fonds der Chemischen Industrie'.

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