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PROCESSING OF PREPROMELITTIN BY SUBCELLULAR FRACTIONS FROM RAT LIVER

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SUMMARY: Prepromelittin, obtained by translation of melittin mRNA in a cell-free system from wheat germ, was incubated with subcellular fractions from rat liver in the presence of deoxycholate. The product formed had the same amino terminal sequence as honeybee promelittin. The processing activity was membrane-bound and was shown to reside in both microsomal and mitochondrial/lysosomal fractions from rat liver.

Melittin, the main component of honeybee venom, is generated from prepromelittin via several enzymatic reactions (1,2). The first of these reactions, the cleavage of the pre-sequence is catalyzed by an intracellular protease which is apparently present in many animal cells and is not species specific. Notably, translation of melittin mRNA in oocytes of Xenopus laevis yields promelittin, while the primary translation product prepromelittin is not detectable (3). This lack of species specificity in the cleavage of pre-sequences is not without precedence. Work from several laboratories has demonstrated that presecretory polypeptides can be processed by microsomal membranes of diverse origin (4-6). In these experiments, the microsomal membranes had to be present during polypeptide synthesis, otherwise completed pre-proteins could not be processed. These findings are in agreement with the prediction of the "signal hypothesis" (7) that in vivo, cleavage of the pre-sequence is an early event which takes place during the vec-

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torial discharge of the growing polypeptide chain into the cisternae of the endoplasmic reticulum.

In this communication we describe a post-translational assay for this pre-protein processing activity, in which prepromelittin is incubated with subcellular fractions from rat liver in the presence of detergent. It is shown that the membrane-bound protease(s) can cleave prepromelittin correctly to yield promelittin. Surprisingly, the highest activity was found in a fraction rich in mitochondria and lysosomes. These results were reported in part at the 11th FEBS Meeting (2).

MATERIALS AND METHODS

Tritiated leucine, proline and alanine (30-50 Ci/mmole) were purchased from The Radiochemical Centre (Amersham). Proteolytic enzymes were obtained from Sigma (St. Louis).

The preparation of total RNA from queen bee venom glands and its translation in a cell-free system from wheat germ (Niblack Food Inc., Rochester, N.Y.) have been described in previous publications (3,8).

Subcellular fractions from liver of Sprague-Dawley rats were prepared by differential centrifugation, as follows. A liver homogenate, 20 % (w/v) in 0.25 M sucrose was centrifuged at 3000 g for 5 min and the mitochondrial/lysosomal fraction collected from the supernatant by centrifugation at 17,000 g for 20 min. The resulting supernatant was further centrifuged at 20,000 g for 30 min and a microsomal pellet was then collected by centrifugation at 100,000 g for 60 min. This pellet was washed once with water to reduce contamination with soluble lysosomal enzymes. For some experiments,the mitochondria and lysosomes were ruptured by a freeze/thaw treatment and the membranes then separated from the soluble fraction by centrifugation at 100,000 g for 60 min. The pelleted membranes were washed once with 0.25 M sucrose. To identify cellular compartments, the following marker enzymes were measured: esterase for microsomes (substrate: p-nitrophenylacetate, ref. 9), acid phosphatase for lysosomes (substrate: o-carboxyphenyl phosphate, ref. 10), and succinate oxidase for mitochondria (assayed polarographically). All activities were measured at 30°C.

The post-translational processing of prepromelittin was assayed in 50 µl incubation mixtures which were 100 mM in Tris/HCl (pH 7.4) and contained 0.2 % sodium deoxycholate, 0.1 - 0.3 mg rat liver protein and 5-10 µl of wheat germ translation system with up to 100,000 counts/min of labeled prepromelittin (about 1 picomole).

Samples were incubated at 37°C for 2 h, subsequently diluted five fold with 0.25 % ammonia and extracted with n-butanol (11). Under these conditions, unreacted prepromelittin accumulates at the interface, while promelittin partitions into the aqueous layer. The latter was further purified by paper

Table 1: Dependence on deoxycholate concentration of the processing reaction

% Processing	% deoxycholate (w/v)					
	0	0.05	0.1	0.2	0.5	1.0
a) Microsomes	3	5	16	20	11	n.d.
b) Mitochondria/ Lysosomes	4	8	41	35	19	11

The processing activity is expressed as the percentage of promelittin formed from prepromelittin labeled with tritiated leucine (see Methods). (n.d., not determined).

chromatography (3) and the region of the chromatogram containing promelittin was eluted. Interface material and promelittin were hydrolyzed with pepsin or chymotrypsin and the resulting digests were fractionated by high voltage paper electrophoresis as described elsewhere (1,11). For quantitative experiments, prepromelittin labeled with [^{3}H] leucine was used and the radioactivity recovered in the peptic fragment Lys-Val-Leu-Thr-Thr-Gly-Leu (residues 50-56 of prepromelittin, 7-13 of melittin) was determined for both the starting material and the cleavage product.

Methods used for the analysis of radioactive fragments have been described previously (1,11).

RESULTS AND DISCUSSION

Characteristics of the cleavage reaction

Incubation of prepromelittin with microsomal or mitochondrial/lysosomal fractions from rat liver yields a product which is soluble in dilute ammonia saturated with n-butanol (see Methods) and co-migrates with honeybee promelittin on paper chromatography (2). As shown in Table 1, the cleavage reaction is dependent on the presence of detergent. Maximal yields, i.e. about 40 % conversion in 2 hours, were obtained at 0.1 - 0.2 % deoxycholate. Triton X-100 could replace deoxycholate, but yields were generally lower. The cleavage reaction gradually ceases after about 3 hours of incubation at 37°C. Addition of

fresh liver fractions did not result in further cleavage. The reason for this incomplete processing may be an inherent mismatch between the insect presecretory protein and the mammalian enzyme(s) or sub-optimal reaction conditions. A similar low yield of post-translational processing in an all-mammalian system was recently reported by Blobel and coworkers (12), who also used the reaction conditions previously reported by us (2). The concentration of deoxycholate in our assays is higher than that which renders the endoplasmic reticulum permeable to polypeptides (less than o.o5 %, ref. 13). These rather high amounts of detergent may be needed to overcome the latency of the enzyme(s). Alternatively, the deoxycholate may be required to keep prepromelittin in a state in which the pre-projunction is sufficiently exposed.

Amino-terminal sequence of the product

We have shown previously that the pro-region of the melittin precursor contains only alanine, proline and acidic residues (1). Chymotryptic hydrolysis of prepromelittin yields a large acidic fragment of 29 residues which begins with alanine^{21} and has the NH_2-terminal sequence: Ala-Ala-Pro-Glu-Pro-Glu-Pro-Ala-Pro-Glu--Pro-Glu-Ala...Queen bee promelittin starts with the second residue (alanine^{22}) of this sequence (1).

Prepromelittin labeled with [^{3}H]proline or [^{3}H] alanine was incubated with the rat liver fractions. Promelittin was isolated, digested with chymotrypsin, and the large acidic fragment was purified. Experiments with [^{3}H] proline (see Fig. 1) and [^{3}H] alanine (data not shown) have demonstrated that this chymotryptic fragment has the partial NH_2-terminal sequence: Ala-Pro-X-Pro--X-Pro-Ala-Pro-X-Pro-X... This must correspond to the amino end of the reaction product, since any cleavage taking place earlier

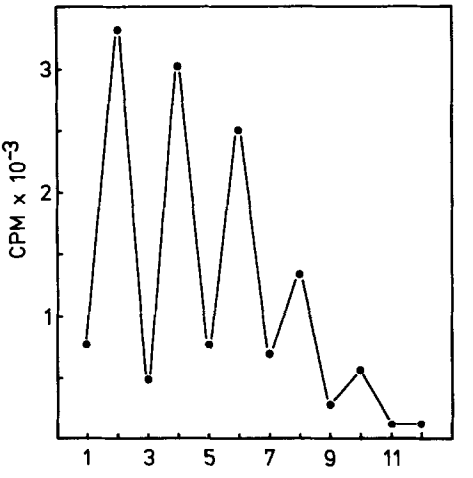

FIGURE 1. Partial sequence of a chymotryptic fragment derived from the amino end of promelittin generated in vitro. Prepromelittin labeled with tritiated proline was incubated with the mitochondrial/lysosomal fraction from rat liver. The resulting promelittin was purified as described in "Methods" and subsequently hydrolyzed with chymotrypsin. The digest was separated by paper electrophoresis (1,11). The fragment with about half the mobility of glutamic acid was eluted and subjected to stepwise Edman degradation. The radioactivity released at each step is plotted against the cycle number.

in the prepromelittin sequence would have yielded a fragment starting with the sequence: Ala-Ala-Pro... Identical results were obtained with promelittin formed in the presence of microsomes or with the mitochondrial/lysosomal fraction. The rat liver fractions are thus able to cleave prepromelittin at the pre-pro--junction between Ala^{21} and Ala^{22}. It is,however, not known whether one or more proteolytic reactions are involved in this processing step.

Subcellular distribution of the processing activity

Early in this investigation the possibility was considered that lysosomal proteases could cleave prepromelittin and by chance yield promelittin. The processing activity of different subcellular fractions was therefore correlated with their content of typical marker enzymes. As shown in Table 2, a markedly

Table 2. Subcellular distribution of the processing activity and of marker enzymes

	Processing activity	Esterase	Acid Phosphatase	Succinate oxidase
Microsomes	9	30,000	10	0
Mitochondria/ Lysosomes				
total	28	6,100	300	70
membranes	30	4,400	60	70
supernatant	6	900	330	0

Processing activity is expressed as percentage of promelittin formed from prepromelittin labeled with tritiated leucine (see Methods). The activities of marker enzymes are given as nanomoles of product/min/mg protein (acid phosphatase, esterase) or as ng atom oxygen/min/mg protein (succinate oxidase). Averages from 2 or 3 experiments are given in the table.

higher yield was obtained with the fraction containing mitochondria and lysosomes than with microsomes. After removal of the soluble proteins the processing activity of the former fraction remained associated with the membraneous part, while the bulk of the lysosomal marker enzyme, acid phosphatase, stayed in the supernatant. The processing activity was therefore not a typical lysosomal hydrolase but rather a membrane-bound enzyme which co-fractionated with the membrane markers succinate oxidase and esterase.

It was somewhat surprising that the highest activity was detected in a fraction containing mainly mitochondria and lysosomes and not in endoplasmic reticulum, as might be expected for a pre-protein processing enzyme. It has yet to be shown, however, whether the activities found in the different fractions and which do yield promelittin with the correct amino end are due

to the same enzyme(s). In addition, the activity in the larger particulate fraction may be associated with the lysosomal or with one of the mitochondrial membranes or with a specialized part of the endoplasmic reticulum (14). Further studies are in progress to establish the distribution and specificity of processing activity between these different membrane fractions.

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