CELL-FREE TRANSLATION OF BOVINE HYPOTHALAMIC mRNA

Synthesis and processing of the prepro-neurophysin I and II

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1. Introduction

Neurophysins are synthesized in neurosecretory neurons of the hypothalamus. They are characterized by their function which is to transport the octapeptides vasopressin and oxytocin along the axons to a storage site in the posterior pituitary [1]. Bovine neurophysin I (Np I) and II (Np II) have been purified and sequenced [2]; they have mol. wt ~10 000. Two groups have identified the tentative precursor to Np I and Np II, respectively, from the hypothalamus of mice and rats [3,4]. The precursors have mol. wts ~20 000 [3,4] and at least one of them is glycosylated [4,5].

The aim of this study was to investigate the cell-free biosynthesis of the reported precursors by translation of mRNA isolated from calf hypothalamus. Also we were interested whether one or both synthesized Np-precursors identified by immunoprecipitation could be cleaved and glycosylated by microsomal membranes as known from studies of other precursor polypeptides [6].

2. Methods

2.1. *Isolation of hypothalamic polysomes and RNA extraction*

Calf hypothalami obtained at the time of slaughter (not later than 10–15 min after sacrifice) were transferred immediately into liquid nitrogen and stored there until use. Hypothalamic tissue (~5 g) was minced and homogenized in 3 vol. buffer A (25 mM Tris–HCl (pH 7.5), 25 mM NaCl, 8 mM $MgCl_2$, 250 mM sucrose, 0.5 mg/ml Na-heparin) by 3 strokes of a Teflon–glass homogenizer. The cell extract was differentially centrifuged at 4000 × *g* for 10 min and at 27 000 × *g* for 30 min. The 27 000 × *g* supernatant fraction contained free polysomes. The pellet of the 27 000 × *g* centrifugation step contained primarily membrane-bound polysomes and was dissolved in buffer A, made 1% (w/w) with respect to both Triton X-100 and Na-deoxycholate and centrifuged at 27 000 × *g* for 30 min; the resulting supernatant contained detached polysomes. Aliquots (20 ml) of free or detached polysome fractions were layered on a discontinuous sucrose gradient containing 6 ml 2.5 M sucrose and 12 ml 1.0 M sucrose in buffer A. The gradients were centrifuged at 100 000 × *g* for 6 h. Polysomes present at the interface of the two sucrose layers were collected and aliquots routinely analyzed by centrifugation in a linear sucrose gradient (15–40%, w/v). The profiles of free and detached polysomes were similar, with most of the ribosomes present as tetra- and pentasomes.

RNA was extracted from free or detached polysomes with SDS/phenol/chloroform [7] and further fractionated by oligo(dT)-cellulose (type T3, Collaborative Res. Inc.) chromatography [8]. The total yield from 1 g (wet wt) of hypothalami was ~60 μg polysomal or 2.5 μg mRNA (1 mg RNA ≃ 25 A_{260} units).

Abbreviations: SDS, sodium dodecyl sulfate; con A; concanavalin A; EDTA, (ethylenedinitrilo)tetraacetic acid; Np, neurophysin; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CNBr, cyanogenbromide

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2.2. *Purification of antisera*

Rabbit antisera raised against porcine Np I and II, respectively, and the antigens were provided by Drs P. Schwandt, W. Richter and G. Neureuther, Munich. Anti porcine Np I or Np II cross-reacted with bovine Np I and Np II, respectively. Antisera were purified by affinity chromatography on columns prepared by linking Np to activated CNBr-Sepharose 4B (Pharmacia manual).

2.3. *Cell-free synthesizing systems and assay conditions*

Wheat germ extract was prepared as in [9,10]. The reaction mixture contained 20 mM Hepes (pH 7.6), 2 mM magnesium acetate, 80 mM KCl, 2 mM dithiothreitol, 0.6 mM spermidine, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 40 μg/ml creatine kinase, 80 μg/ml mRNA, 25 μM 19 unlabeled amino acids and 200 μCi/ml of either [^{35}S]methionine (spec. act. > 500 Ci/mmol), [^{35}S]cysteine (spec. act. > 500 Ci/mmol) or [^{3}H]leucine (spec. act. > 100 Ci/mmol).

The rabbit reticulocyte lysate assay system (New England Nuclear) contained (per ml): 20 μg mRNA and 800 μCi of either [^{35}S]methionine or [^{35}S]cysteine or 400 μCi [^{3}H]leucine (New England Nuclear).

Table 1
Translation of hypothalamic mRNA in reticulocyte lysate and wheat germ system

Cell-free system	Labeled amino acid	mRNA	Incorp. (cpm/2 μl)
Wheat germ		–	3800
		+	10 500
	[^{3}H]Leucine		
Reticulocyte		–	500
lysate		+	5500
Wheat germ		–	12 500
		+	53 000
	[^{35}S]Methionine		
Reticulocyte		–	25 000
lysate		+	125 000
Wheat germ		–	25 000
		+	42 000
	[^{35}S]Cysteine		
Reticulocyte		–	50 000
lysate		+	149 000

Conditions for mRNA translation see section 2. Trichloroacetic acid precipitation of the translation products was carried out as in [9]. The data shown are not comparable in respect to their absolute values because of the different amounts of radioactive amino acids present during translation in both cell-free systems (see section 2). [^{35}S]Cysteine-labeled polypeptides were not alkylated before trichloroacetic acid precipitation

3. Results and discussion

3.1. *Translation of mRNA isolated from hypothalamic polysomes*

Hypothalamic mRNA isolated from free or detached polysomes was translated in cell-free systems prepared from wheat germ or reticulocyte lysate. mRNA from free polysomes showed slightly better amino acid incorporation per μg RNA than that from detached polysomes. Maximal amino acid incorporation was achieved in the presence of 0.5–2.0 μg mRNA/25 μl assay within 30–40 min at 37°C (reticulocyte system) or 25°C (wheat germ system). Table 1 shows that [^{3}H]leucine incorporation was stimulated up to 11-fold by hypothalamic mRNA in the reticulocyte system. In the same system stimulation of [^{35}S]methionine or [^{35}S]cysteine incorporation into trichloroacetic acid-insoluble material was significantly lower. Generally the wheat germ system was less efficient in translating hypothalamic mRNA. Analysis of the translation products by SDS gel electrophoresis followed by fluorography [11] revealed numerous polypeptides synthesized in the reticulocyte system (fig.1, lane 7) whereas in the wheat germ system particularly the high molecular weight polypeptides were less evident (fig.1, lane 1).

3.2. *Analysis of the translation products by immunoprecipitation*

mRNA from free or detached polysomes was assayed, although most experiments were carried out with detached polysome mRNA which showed increased synthesis of neurophysin-like polypeptides. Purified anti Np I or anti Np II was added to the assay tubes after translation and immunoprecipitates were analyzed by SDS–gel electrophoresis. Three polypeptides with mol. wt 21 000 ± 10% (α), 18 000 ± 10% (β) and 16 500 ± 10% (γ) could be identified in both translation systems (fig.1). In the wheat germ system the 18 000 mol. wt polypeptide

appeared as a minor band detectable only upon prolonged exposure. The specificity of the immunoprecipitation was analyzed by competition experiments with excess amounts of Np I or II (fig.1, lane 2B, 3B, 4B, 5B, 6B). The results clearly showed that authentic Np and the three cell-free synthesized peptides competed effectively for the same Np-specific antiserum. No competition was observed with bovine serum albumin; the 3 polypeptides were not precipitated with non-immune serum or in the absence

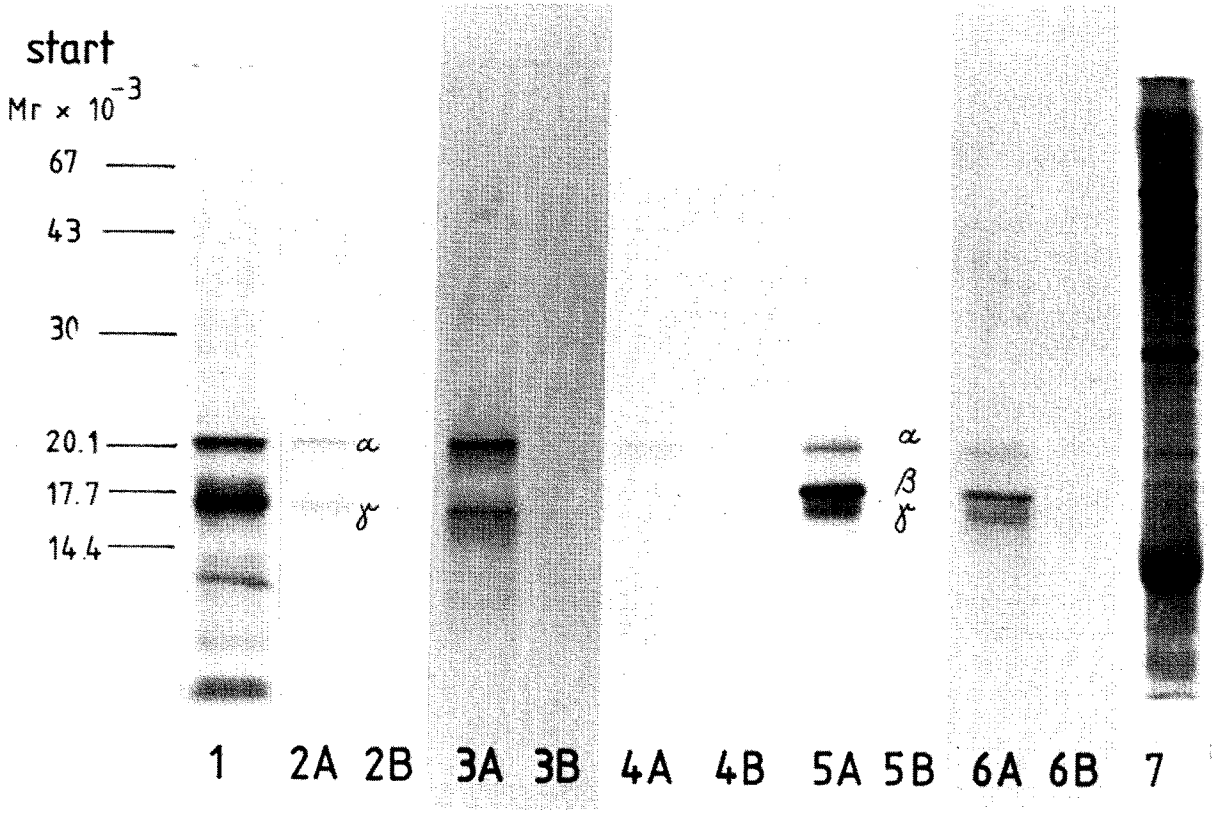

Fig.1. SDS–polyacrylamide gel electrophoresis of total and immunoprecipitated polypeptides synthesized by translation of hypothalamic mRNA from detached polysomes. After incubation, 25 μl aliquot of the reaction mixture incubated with either [^{35}S]cysteine, [^{35}S]methionine or [^{3}H]leucine was diluted with 3 vol. 10 mM Na-phosphate buffer (pH 7.6) containing 1 mM EDTA, 1% Triton X-100 (w/v), 200 units/ml Antagosan (Behring Werke, Marburg); 10 μg purified anti-Np measured at A_{280} (1.4 A_{280} units ≃ 1 mg) were added. After incubation for 20 h at 4°C and centrifugation at 15 000 × *g* for 15 min indirect immunoprecipitation was carried out using *Staphylococcus aureus* immunoabsorbant (kindly provided by Drs R. Jaenisch and P. Nobis, Hamburg; [12]). The immunoprecipitates were electrophoresed on discontinuous SDS–polyacrylamide gels [13] containing 15% acrylamide (at constant 5 W). Gels were dried and fluorographed [11]. Wheat germ system (lanes 1–4B): lane 1, total cell-free products synthesized in 10 μl assay mixture with [^{35}S]cysteine; lanes 2A,3A,4A, immunoprecipitates derived from [^{35}S]cysteine (lane 2A), [^{3}H]leucine (lane 3A) or [^{35}S]methionine (lane 4A) labeled translation products in lanes 2B,3B,4B the translation assay was identical to lanes 2A,3A,4A, respectively, except that immunoprecipitation was performed in the presence of excess of porcine Np II as competing peptide. Reticulocyte system (lanes 5A–7): lanes 5A,6A, immunoprecipitates derived from [^{35}S]cysteine (lane 5A) and [^{3}H]leucine (lane 6A) labeled translation products; lanes 5B,6B, same as lanes 5A,6A except that immunoprecipitation was in the presence of porcine Np II; lane 7, total cell-free products synthesized in 5 μl translation mixture with [^{35}S]cysteine. The following proteins were used for molecular weight determination: bovine serum albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soy bean trypsin inhibitor (20 100), myoglobin (17 200), α-lactalbumin (14 400).

of added mRNA (not shown). The data strongly suggested that the 3 polypeptides synthesized by translation of hypothalamic mRNA contained amino acid domains very similar to or identical with authentic neurophysins. In both cell-free systems [^{3}H]leucine and [^{35}S]cysteine were incorporated into the 21 000, 18 000 and 16 500 mol. wt polypeptide whereas [^{35}S]methionine was present significantly only in the 21 000 polypeptide (fig.1, lane 4A). Lack of methionine in the 18 000 and 16 500 mol. wt immunoreactive products suggests that this amino acid contributes little to the amino acid composition of the two polypeptides. However, methionine was incorporated in the NH_2-terminal position of all 3 polypeptides when *N*-formyl-[^{35}S]methionyl-tRNA$_i^{Met}$ was used in the translation assay (not shown).

Recent in vivo studies [5] suggest that Np I and Np II are synthesized as precursor polypeptides that differ in their pI and molecular weights. Since anti-Np I serum cross-reacted with Np II and vice versa the cell-free synthesized Np-precursors could not be correlated to Np I or Np II. The finding that 3 polypeptides could be immunoprecipitated suggested that one of them may be an incomplete immunoreactive translation product. Based on the processing experiments shown below we assume that the 21 000 mol. wt polypeptide is one of the two Np-precursors. That the two smaller polypeptides (18 000 and 16 500 mol. wt) are derived from the 21 000 mol. wt product by proteolytic cleavage seems to be unlikely since the ratio of the amount of the 3 polypeptides synthesized did not change during the time of incubation (not shown). Alternatively the 18 000 and 16 500 mol. wt polypeptides could result from pre-termination events and represent a premature 21 000 mol. wt polypeptide. Cotranslation experiments with microsomal membranes indicated, however, that most likely the 18 000 mol. wt immunoreactive translation product is the second Np-precursor and that the 16 500 mol. wt polypeptide is a premature polypeptide probably obtained by a pre-termination event.

3.3. *Processing of the precursors by microsomal membranes from dog pancreas*

Many secretory polypeptides are synthesized as prepro-forms which contain a hydrophobic signal peptide essential for vectorial discharge of the peptide into the intravesicular space [6]. The signal peptide is then usually cleaved off by proteolytic enzyme(s) in the microsomal fraction yielding the proform of the polypeptide. By analogy, the immunoreactive translation products shown in fig.1 should correspond to the preproforms of neurophysin I and II, respectively. They should be processed into proforms when

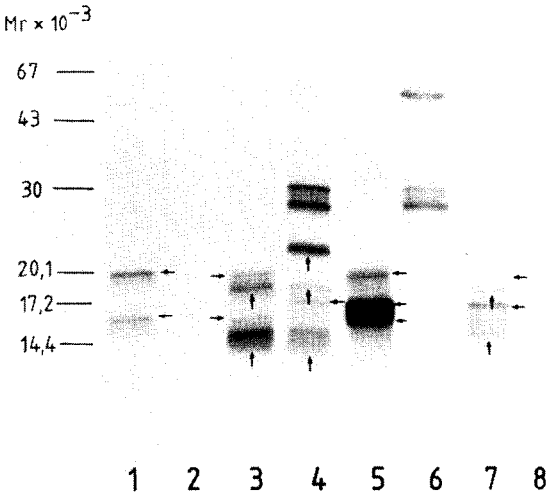

Fig.2. Processing of the immunoprecipitable translation products in the presence of microsomal membranes from dog pancreas or tunicamycin-treated ascites tumor cells. Crude dog pancreas microsomal membranes were kindly provided by Dr B. Dobberstein, Heidelberg and further purified [14]. Preparation of membranes from tunicamycin-treated ascites tumor cells was carried out as in [15]. Tunicamycin was kindly provided by Lilly Res. Lab. through Dr D. Rifkin, New York. mRNA from hypothalamic detached polysomes was translated in cell-free wheat germ (lane 1–3) or reticulocyte lysate system (lane 4–8) containing [^{35}S]cysteine in the absence of membranes (lanes 1,5) or in the presence of microsomal membranes from either dog pancreas (8 A_{260} units/ml; lanes, 3,4) or tunicamycin-treated ascites tumor cells (3 A_{260} units/ml; lane 7). Immunoprecipitation, SDS–gel electrophoresis and fluorography was carried out as in fig.1. In lanes 2,6,8 experimental conditions were as in lanes 3,4,7 except that excess of Np II was present during immunoprecipitation. The higher molecular weight polypeptides (> 25 000) observed in the reticulocyte lysate system were only present when translation was assayed with dog pancreas microsomal membranes; the polypeptides were unspecifically precipitated which is indicated by the competition experiments with Np II (lane 6). The vertical arrows indicate processed, the horizontal arrows non-processed immunoreactive translation products. For molecular weight markers see fig.1.

the protein synthesizing system is supplemented with microsomal membranes. As indicated in fig.2, two additional immunoreactive translation products appeared with mol. wt 19 000 ± 10% and 15 500 ± 10% (lane 3) in the membrane supplemented wheat germ system. From predictions of the signal hypothesis and by analogy with [6] we interpret these data such that in the presence of microsomal membranes the 21 000 mol. wt prepro-form was converted into a 19 000 mol. wt pro-form, the 16 500 mol. wt polypeptide into a 15 500 mol. wt product. By comparison the 21 000 and 16 500 mol. wt polypeptides were similarly processed in the reticulocyte system (lane 4). An additional immunoreactive translation product of 23 000 mol. wt appeared in the membrane supplemented reticulocyte (lane 4), not in the wheat germ system (lane 3). The 23 000 mol. wt but not the other immunoreactive products was retained on con A–Sepharose columns from which it could be eluted by α-methylmannoside suggesting that the 23 000 mol. wt product was glycosylated. It has been shown before that in cell-free protein synthesizing systems translation of mRNA for glycoproteins results in synthesis of glycosylated polypeptides provided microsomal membranes are present during translation [6]. The 23 000 mol. wt polypeptide was not observed (lane 7) when cotranslation was performed with microsomal membranes prepared from tunicamycin-treated ascites tumor cells [15]. Tunicamycin is known to inhibit synthesis of carbohydrate–lipid intermediates that normally serve as donors in the formation of dolichol-linked oligosaccharides, which in turn are prerequisites of the microsomal membrane-dependent glycosylation of polypeptides [16]. As expected tunicamycin-treated membranes were inactive in producing the 23 000 mol. wt polypeptide whereas they were active in processing the 21 000 mol. wt precursor; processing of the 18 000 mol. wt polypeptide also appeared, however the processed product migrated closely to the 16 500 mol. wt polypeptide and therefore was only poorly resolved in the autoradiogram (lane 7).

When microsomal membranes from dog pancreas were added to the cell-free wheat germ system the glycosylated 23 000 mol. wt polypeptide was not observed although in parallel experiments with mRNA from pituitary the cell-free synthesized and immunoprecipitated pro-opiocortin was glycosylated (unpublished). The absence of the 23 000 mol. wt polypeptide in the wheat germ system may be explained by lack of the 18 000 mol. wt precursor (fig.2, lane 1). It also supports the notion that the 18 000 mol. wt polypeptide synthesized in the reticulocyte lysate system is the prepro-form of one of the neurophysins from which the 23 000 pro-form is derived by processing and glycosylation.

These data may be consistent with the in vivo isolation [5] of two polypeptides from rat hypothalamus that are believed to be the precursors not only of the neurophysins but also of oxytocin and vasopressin. The two prohormones differ in their molecular weights but only one of them is glycosylated [5]. By analogy the cell-free translation products of 21 000 and 18 000 mol. wt reported here, could also be the prepro-hormones of either oxytocin or vasopressin. The processed 19 000 and 23 000 mol. wt polypeptides should be prohormones of the two octapeptides. We are presently analyzing the cell-free translation products utilizing antisera raised against oxytocin and vasopressin which should allow us to identify the precursors to these two hormones.

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