Human neuropeptide Y, somatostatin and vasopressin precursors identified in cell-free translations of hypothalamic mRNA

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Messenger mRNA has been prepared from post mortem human hypothalami and translated in a cell-free system. Using specific antibodies, biosynthetic precursors have been identified to neuropeptide Y (12 kDa), somatostatin (15 kDa) and vasopressin/neurophysin (19 kDa).

Human hypothalamus *Neuropeptide Y* *Somatostatin* *Vasopressin* *Neurophysin* *Hormone precursor*

1. INTRODUCTION

The assignment of neurotransmitter activity to a number of brain neuropeptides implies a significant involvement of these peptides in mental function. To understand the regulation of such peptide hormones it is a prerequisite to gain a detailed breakdown of their biosynthetic pathways. However, for obvious reasons there is little information on the initial stages of biosynthesis for human brain peptide precursors. Post mortem autolytic activity may rapidly degrade the early precursors and the mRNA encoding them. An exception has been the recent description of the cell-free synthesized precursor to human luteinizing hormone releasing hormone [1].

In part, this deficit can be circumvented by the isolation of genes from human genomic libraries (e.g., [2]); the precursor structures derived from these DNA sequences, however, remain hypothetical until corroboratory data is obtained directly on the mRNA or protein precursor products of these genes.

Here we report the isolation and cell-free translation of human post mortem hypothalamic mRNA and the identification of the human prepro-hormones for neuropeptide Y, somatostatin and vasopressin.

2. EXPERIMENTAL

2.1. *Preparation of mRNA*

Human hypothalami were obtained at autopsy within 4 h (male, 44 years) or 12 h (male, 76 years) of death from cases where the cause of death was unrelated to brain function (both myocardial infarctions). Hypothalami were immediately homogenized in 6 vols of a solution containing 4 M guanidinium thiocyanate (Bethesda Research Laboratories, Neu Isenburg, FRG) and 1 M β-mercaptoethanol. This homogenate is stable at room temperature in sealed containers for several days.

Total RNA was isolated by centrifugation through CsCl [3] and enriched for poly(A)-containing sequences by oligo(dT)–cellulose chromatography [4]. A single human hypothalamus yields approx. 75 μg poly(A)-rich RNA.

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2.2. *Cell-free translation and immunoprecipitation of specific products*

One μg poly(A)-rich RNA was translated in 50-μl reaction mixtures of rabbit reticulocyte lysate as described in [4], using as radiolabel, 100 μCi [^{35}S]methionine and 40 μCi [^{35}S]cysteine (both from New England Nuclear, translation grade) per reaction. Whereas cysteine is present in somatostatin, vasopressin and its associated neurophysin, methionine appears to be the only suitable label to identify human neuropeptide Y [5]. After 1 h incubation at 37°C, when trichloroacetic acid precipitation indicated a maximum specific incorporation of ~10^6 cpm/μg hypothalamic poly(A)-rich RNA, translation mixtures were supplemented by 3 volumes of a buffer containing 10 mM Na-phosphate (pH 7.6), 2% (v/v) Trasylol, 1 mM EDTA. Five mM cysteine, 5 mM methionine, 2 mM dithiothreitol, 1% (w/v) Nonidet P-40, 1% (w/v) sodium deoxycholate and 0.3% (w/v) SDS. To reduce nonspecific immunoprecipitation, the above translation solutions were preincubated for 30 min at 4°C with 50 μg rabbit preimmune IgG and 100 μl of 10% (w/v) suspension of fixed and washed staphylococcal immunoadsorbent (IgSorb, the Enzyme Center, Boston, USA) and centrifuged. The supernatant fractions were then supplemented with 50 μg peptide-specific antibodies (see below) as IgG fractions obtained by 40% saturated ammonium sulfate precipitation. Where indicated, as competitive controls, the antibodies were quenched with an excess (10–20 μg) of the respective unlabeled peptide antigen. After overnight incubation at 4°C the resultant immune complexes were isolated using staphylococcal immunoadsorbent, as described [4], and subjected to electrophoresis on 15% polyacrylamide-SDS gels followed by fluorography.

2.3. *Antisera*

Anti-vasopressin, anti-somatostatin-14 (both from Ferring, Kiel, FRG), and anti-somatostatin-28 (S309, by courtesy of Dr R. Benoit) have been characterized and described elsewhere [6,7]. Anti-neurophysin (by courtesy of Dr A. Weindl) appears to recognize predominantly the vasopressin-associated neurophysin among the products of rat and bovine hypothalamic translations (Ivell, unpublished). All these antisera have been demonstrated to be highly specific for their respective antigenic sequences. Identification of precursors from other mammals using these antisera has been confirmed by tryptic peptide mapping or subsequent sequence analysis [8,9]. Anti-neuropeptide Y was raised in rabbits using porcine neuropeptide Y (by courtesy of Dr K. Tatemoto) conjugated to bovine serum albumin using carbodiimide. Neuropeptide Y antiseraum no.11 was used here; this antiserum recognizes the intact neuropeptide Y molecule [10].

3. RESULTS AND DISCUSSION

In vitro translation is a reliable indicator of the quality of an mRNA preparation. The hypothalamic specific incorporation of radioactive amino acids programmed by the human mRNA was no different from that observed with hypothalamic mRNA of rat or bovine origin where the delay from death to RNA preparation is only 1 min or 20–40 min, respectively (unpublished).

Electrophoresis of total translation products shows a number of high molecular mass proteins (fig.1, lane 2), implying minimal RNA degradation. More critical than the delay between death and dissection is probably the speed with which RNase activity instigated by tissue damage or contamination can be eliminated by, for example, guanidinium thiocyanate homogenization.

3.1. *The human precursor to neuropeptide Y*

Previous studies have indicated that neuropeptide Y-like immunoreactivity can be detected in substantial amounts in the human forebrain [11]. The presence of large amounts of neuropeptide Y, somatostatin and vasopressin in human hypothalamus suggested that this region would be appropriate for these studies. The demonstration that human neuropeptide Y contains a methionine residue [5] indicated that this amino acid would be a suitable radiolabel for incorporation into a human NPY precursor. Recent work [12] using recombinant DNA techniques has confirmed the methionine residue in NPY and demonstrated further methionine and cysteine in the signal sequence of the human precursor [12].

Fig.1, lane 3, indicates that antisera raised against human neuropeptide Y are able to select a methionine and/or cysteine-labeled polypeptide of apparent molecular mass of 12 kDa which is not

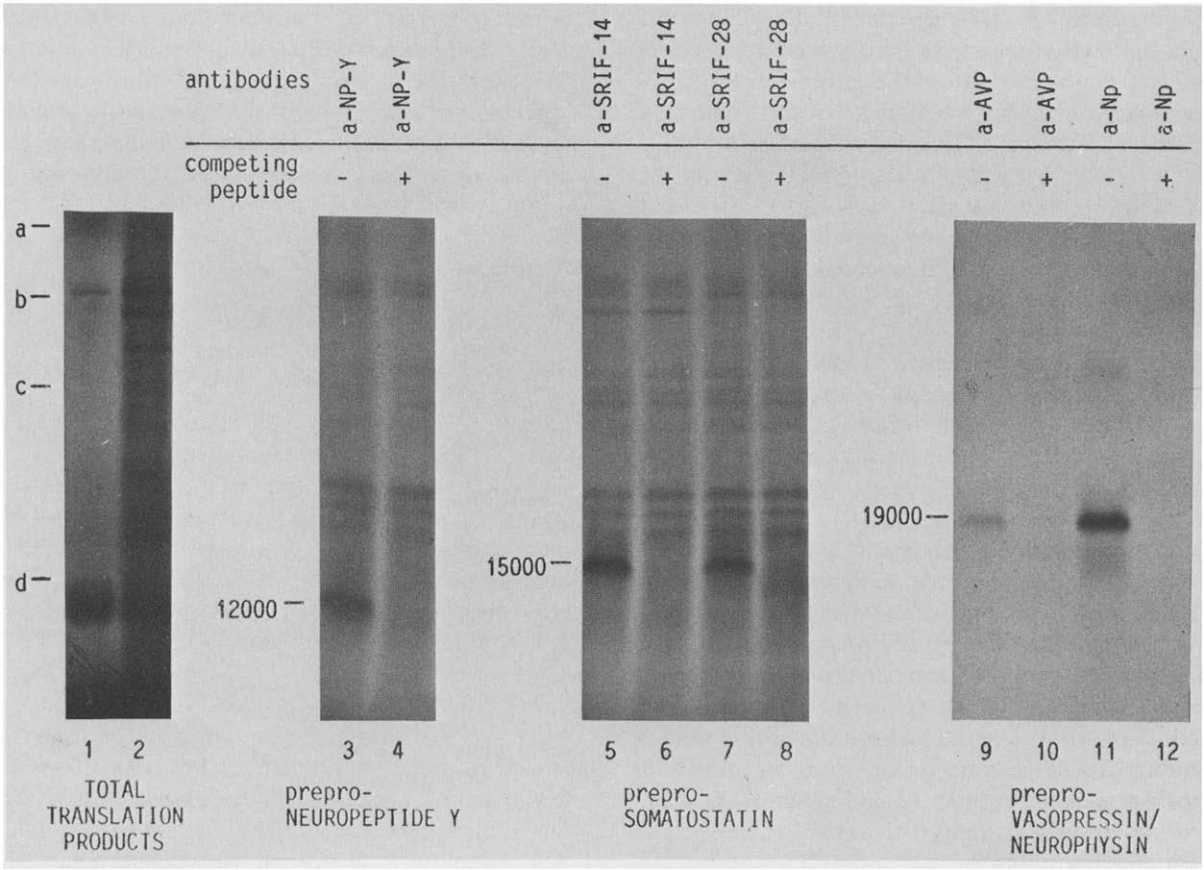

Fig.1. Immunoprecipitated precursors to neuropeptide Y, somatostatin and vasopressin/neurophysin among translation products programmed by human hypothalamic mRNA. Lanes 1, 2: 2 μl of total translation mix before immunoprecipitation, in the absence (lane 1) and presence (lane 2) of human hypothalamic mRNA. Lanes 3–12: translates immunoprecipitated with anti-neuropeptide Y (a-NP-Y, lanes 3,4), anti-somatostatin-14 (a-SRIF-14, lanes 5,6), anti-somatostatin-28 (a-SRIF-28, lanes 7,8), anti-vasopressin (a-AVP, lanes 9,10) and anti-neurophysin (a-Np, lanes 11,12). Unlabeled antigen has been added where indicated as competitive control. Lanes 1,2,9–12 are after only 4 days exposure to film, whereas lanes 3–8 are after 4 weeks exposure. Figures represent the apparent molecular masses (kDa) of the identified precursors. Calibration proteins: (a) bovine serum albumin, 69; (b) ovalbumin, 46; (c) carbonic anhydrase, 30; (d) lysozyme, 14.3. The human hypothalamic mRNA used for the experiments illustrated was derived from the 44-year-old male. Similar results not shown were obtained for the other mRNA preparation.

present on pretreating antisera with unlabeled antigen (lane 4). The other much weaker bands visible in this figure and shared in common between most lanes are a consequence of the long exposure time (4 weeks) required to visualize the evidently rare neuropeptide Y precursor. The apparent size of this prepro-neuropeptide Y is in good accord with the amino acid sequence which has been derived from the recently characterized human gene for neuropeptide Y [12]. This structure predicts a precursor of around 11 kDa (10.839 kDa) which is within the limit of resolution of the SDS gel system used here.

3.2. *The human precursor to somatostatin*

Although the human gene for somatostatin has been sequenced and located on chromosome 3 [2,13], there is no direct information as to the mRNA or protein product of this gene in the human hypothalamus. Using antibodies which recognize either the C-terminus of somatostatin-14 (fig.1, lane 5; control, lane 6) or the N-terminus of

somatostatin-28 (lane 7; control, lane 8), shows that both peptides are evidently present in a common precursor of 15 kDa. This prepro-form is consistent with that predicted by the human gene sequence, where the somatostatin-28 molecule, and also the internal somatostatin-14 sequence, occupy the C-terminus of the precursor. It is thus also comparable to the hypothalamic prepro-somatostatins recently described for other mammalian species [4,9].

3.3. *The human precursor to vasopressin*

The in vivo and in vitro synthesized precursors to nonapeptide vasopressin have been well characterized [14,15] and the sequences of both mRNA and gene have been elaborated [8,16]. The hormone precursor includes vasopressin immediately following the signal peptide which cues the nascent polypeptide into the endoplasmic reticulum. The vasopressin sequence is followed by the vasopressin-associated neurophysin found together with the hormone in neurosecretory granules. The C-terminus of the precursor is occupied by a glycopeptide whose function is not yet known. All these data relate to the rat or bovine hormone. In the human, a similar structure seems probable, since proteins of 20–30 kDa, believed to contain both vasopressin and neurophysin sequences, have been observed in human pituitary extracts [17] and in lung carcinomas [18]. Furthermore, a C-terminal glycopeptide-like molecule has been identified in human hypothalamus and pituitary, and its histological distribution parallels exactly that of vasopressin and neurophysin [19].

Cell-free translation of mRNA from the human hypothalamus (fig.1, lanes 9–12) indicates heavy labeling of a precursor of 19 kDa which contains antigenic determinants to both arginine vasopressin (lane 9; control, lane 10) and neurophysin (lane 11; control, lane 12). Since no endoplasmic reticular membranes are present, this polypeptide represents the unglycosylated prepro-hormone. This result agrees well not only with the evidence from other species but also with the limited human data [17,18] and implies a similar common precursor molecule to both vasopressin and its associated neurophysin.

This report confirms the biosynthesis of three peptide hormones, neuropeptide Y, somatostatin and vasopressin, in the human hypothalamus and describes the longer polypeptides which are their precursors. The availability of human hypothalamic mRNA of good quality should allow the exploration of further brain biosynthetic pathways.

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