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Immunological identification of a common precursor to arginine vasopressin and neurophysin II synthesized by *in vitro* translation of bovine hypothalamic mRNA

(signal sequence/polyprotein/processing/core glycosylation/tunicamycin)

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ABSTRACT mRNA from membrane-bound polysomes of bovine hypothalamus was translated in an mRNA-dependent cell-free system from reticulocyte lysate or wheat germ. The translation products were identified by immunoprecipitation with antibodies to either neurophysin II or arginine vasopressin followed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. An immunoreactive polypeptide was obtained with an apparent M_r of 21,000. Sequential immunoprecipitation studies indicated that the M_r 21,000 product is a common precursor to neurophysin II and arginine vasopressin. The specificity of the immunoprecipitation was demonstrated by competition with excess amounts of unlabeled neurophysin II or arginine vasopressin; little or no competition was observed with unlabeled neurophysin I or oxytocin. Processing experiments with microsomal membranes from dog pancreas or tunicamycin-treated ascites tumor cells showed that the M_r 21,000 polypeptide is the prepro form. It was converted to a pro form with M_r 19,000 suggesting a pre sequence of approximately 15 amino acids. The M_r 19,000 polypeptide was core-glycosylated to an apparent M_r of 23,000, indicating that the neurophysin II-arginine vasopressin precursor is a glycopolypeptide.

Oligopeptides such as the bacterial antibiotics gramicidin and tyrocidin are synthesized in a nonribosomal fashion on polyenzyme templates (1, 2). Reports that oligopeptides of hypothalamus from mammals also might be synthesized by a nonribosomal mechanism are still controversial (3–5). *In vitro* biosynthesis of the hypothalamic nonapeptide hormones vasopressin and oxytocin has not yet been resolved. Although Sachs (6) suggested more than a decade ago that vasopressin may be synthesized by a ribosome-dependent mechanism via a precursor polypeptide, only recently a tentative glycopolypeptide precursor with an apparent M_r of 20,000 has been isolated from the hypothalamus of rats and mice (7–9). This precursor gave rise to a vasopressin-like peptide after trypsinization (7). In addition the precursor crossreacted with antibodies raised against one of the hypothalamic cysteine-rich neurophysins (8, 9) which is known to transport vasopressin to its storage site in the posterior pituitary (10).

If the precursor hypothesis is valid it should be possible to demonstrate biosynthesis of a vasopressin-neurophysin precursor directly by translation of hypothalamic mRNA in cell-free systems. Although a number of groups have reported the synthesis of neurophysin precursors in cell-free systems (11–13), identification of vasopressin-like sequences within the reported precursors has been lacking. The present communication reports mRNA-directed synthesis of a common precursor to arginine vasopressin (AVP) and neurophysin II and its processing and core glycosylation; specific antibodies were used for identification.

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MATERIALS AND METHODS

Bovine neurophysin I (Np I) and neurophysin II (Np II) and rabbit antisera to Np I and Np II were purchased from Bioproducts (Brussels); according to the supplier the antisera against Np II were passed over a Np I-Sepharose column to improve the specificity of anti-Np II. The same was done for anti-Np I. The supplier claimed that this procedure reduced crossreactivity of the neurophysin antisera to less than 0.5% as judged by radioimmunoassay. Both these and the rabbit antiserum against AVP (Ferring, Kiel) were precipitated with 28 g of ammonium sulfate per 100 ml. According to Ferring, there is no crossreaction between anti-AVP and oxytocin or lysine vasopressin and less than 30% with vasotocin; anti-AVP does not discriminate between oxidized and reduced AVP. Hypothalamic mRNA was isolated as reported (13). The cell-free wheat germ system was prepared according to published procedures (13). The reticulocyte lysate assay system came from New England Nuclear. EDTA-stripped microsomal membrane fractions from dog pancreas (14) were kindly provided by B. Dobberstein (Heidelberg). Microsomal membranes from ascites tumor cells (15, 16) treated with tunicamycin (Lilly) and immobilized membranes from *Staphylococcus aureus* (17) were prepared as reported.

RESULTS

Identification of the Immunoreactive Products. mRNA isolated from detached polysomes of bovine hypothalami was translated in a cell-free system from reticulocyte lysate or wheat germ in the presence of [^{35}S]cysteine. Purified antibodies raised against neurophysin II (anti-Np II) or arginine vasopressin (anti-AVP) were used to identify the translation products. The immunoreactive products precipitated with *S. aureus* immunoabsorbant were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. With anti-Np II, two products with apparent M_r of 21,000 and 18,000 were obtained, whereas with anti-AVP only the M_r 21,000 product was found (Fig. 1, lanes 1 and 2). The M_r 18,000 product was barely detectable when mRNA was translated in the cell-free wheat germ system. At present it is not possible to decide whether the M_r 18,000 product represents another discrete neurophysin II-like polypeptide (18) or whether it is a premature M_r 21,000 polypeptide derived from a pre-termination event.

The specificity of the immune reactions was demonstrated

Abbreviations: Np I, bovine neurophysin I; anti-Np I, antibodies against Np I raised in rabbits; Np II, bovine neurophysin II; anti-Np II, antibodies against Np II raised in rabbits; AVP, arginine vasopressin; anti-AVP, antibodies raised against AVP in rabbits.

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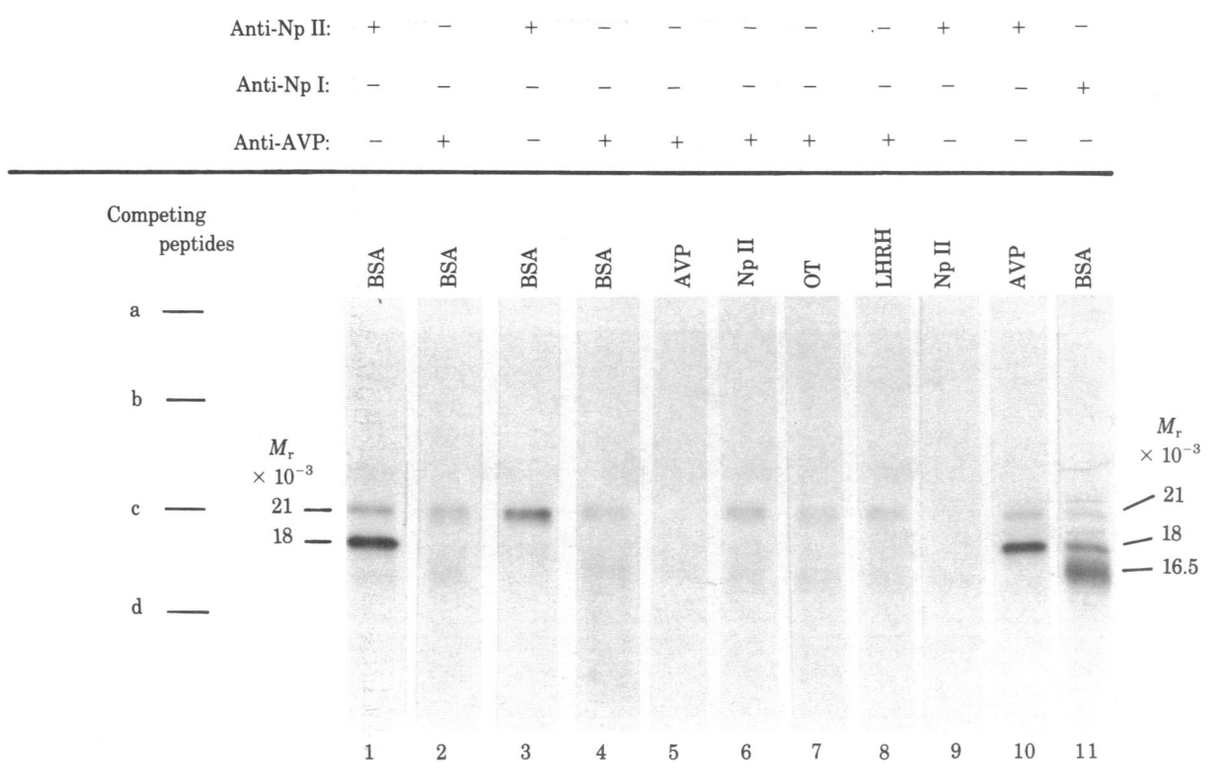

FIG. 1. Gel electrophoresis of the Np II-AVP precursor after synthesis and immunoprecipitation. Bovine hypothalamic mRNA (0.5 μg) prepared from detached polysomes and purified by oligo(dT)-cellulose chromatography (13) was translated in the wheat germ system (lanes 3 and 4) or the reticulocyte lysate system (remaining lanes) in a total volume of 25 μl [10 μl of lysate, 2 μl of translation cocktail, 0.5 μl of 32.5 mM $Mg(OAc)_2$, 2 μl of 1 M KOAc, and 20 μCi of [^{35}S]cysteine]. After incubation at 37°C the mixture was diluted with 3 vol of cold 10 mM Na phosphate, pH 7.6/1 mM EDTA/1% Triton X-100 containing 200 units of Antagosan (Behring, Marburg) per ml. For immunoprecipitation, 10 μg of anti-Np I or anti-Np II IgG or 20 μg of anti-AVP IgG (1.4 A_{280} units = 1 mg) were added and incubated at 4°C for 20 hr. Nonspecific aggregates were removed by centrifugation at 15,000 × *g* for 15 min; the supernatant fraction was subjected to indirect immunoprecipitation with *S. aureus* immunoabsorbant (13, 17). After washing by three cycles of centifugation (8000 × *g*, 2 min) and resuspension in 20 mM Tris·HCl, pH 7.6/1 mM EDTA/500 mM NaCl/0.5% Triton X-100, the antigen–antibody complexes were dissociated from the staphylococci by boiling for 3 min in 62.5 mM Tris·HCl, pH 6.8/5% (wt/vol) sodium dodecyl sulfate/10% (wt/vol) glycerol/4% (vol/vol) 2-mercaptoethanol and subsequently subjected to sodium dodecyl sulfate/15% polyacrylamide gel electrophoresis. The dried slab gels were fluorographed. [For the total translation products (prior immunoprecipitation), see ref. 13.] In this and the following fluorogram, lanes were derived from different gels and aligned according to the position of marker proteins (a, ovalbumin, M_r 43,000; b, carbonic anhydrase, 30,000; c, soy bean trypsin inhibitor, 20,000; d, α-lactalbumin, 14,400). Where indicated, the immunoprecipitation was carried out in the presence of excess (10 μg per assay) of unlabeled competing peptide [bovine serum albumin (BSA), AVP, Np II, oxytocin (OT), or luteinizing hormone-release hormone (LHRH)].

by competition experiments. Excess AVP effectively inhibited binding of the M_r 21,000 product to AVP specific antibodies (Fig. 1, lane 5); no inhibition was observed with excess Np II (lane 6), oxytocin (lane 7), or LHRH (lane 8). On the other hand excess Np II (lane 9) but not AVP (lane 10) inhibited binding of the M_r 21,000 and 18,000 polypeptides to anti-Np II. Np I was significantly less potent as a competitor than Np II although at high concentrations Np I was also inhibitory (not shown). Apparently, anti-Np II did not discriminate completely between the two neurophysins. Similarly, in the reverse experiment anti-Np I crossreacted with the M_r 21,000 and 18,000 precursors, although it predominantly reacted with a polypeptide of M_r 16,500 (lane 11). The M_r 16,500 polypeptide has been identified as the precursor to Np I and oxytocin (19).

To determine whether both anti-Np II and anti-AVP were recognizing the same polypeptide, sequential immunoprecipitation was performed. The immunoprecipitate obtained with anti-Np II was sedimented and the supernatant fraction subsequently was supplemented with anti-AVP. A similar experiment was performed in the reverse order. The results (Table 1) show that the M_r 21,000 product had been removed from the assay mixture by the first immunoprecipitation step; little or no immunoreactive material was detectable in the second immunoprecipitation step, implying that the M_r 21,000 polypeptide is a common precursor to Np II and AVP. In some exper-

Table 1. Sequential immunoprecipitation of the Np II-AVP precursor

Antibodies added		Total cpm in Np II-AVP precursor	
1st step	2nd step	1st step	2nd step
Anti-Np II	Anti-AVP	1192	192
Anti-AVP	Anti-Np II	1044	290

Translation in the reticulocyte lysate system, immunoprecipitation, and gel electrophoresis were carried out as described in Fig. 1. For sequential immunoprecipitation, 25 μl of the translation assay mixture was subjected to an initial immunoprecipitation step with anti-Np II. The immunoprecipitation was collected by centrifugation; the supernatant fraction of this step was supplemented with anti-AVP. A similar experiment was performed in the reverse order using a 25-μl aliquot of the translation mixture and starting the immunoprecipitation with anti-AVP; the immunoreactive material was precipitated by centrifugation and the supernatant fraction supplemented with anti-Np II. The immunoreactive material was analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography. The radioactive polypeptide corresponding to the Np II-AVP precursor was cut out of the gels and measured in a liquid scintillation counter.

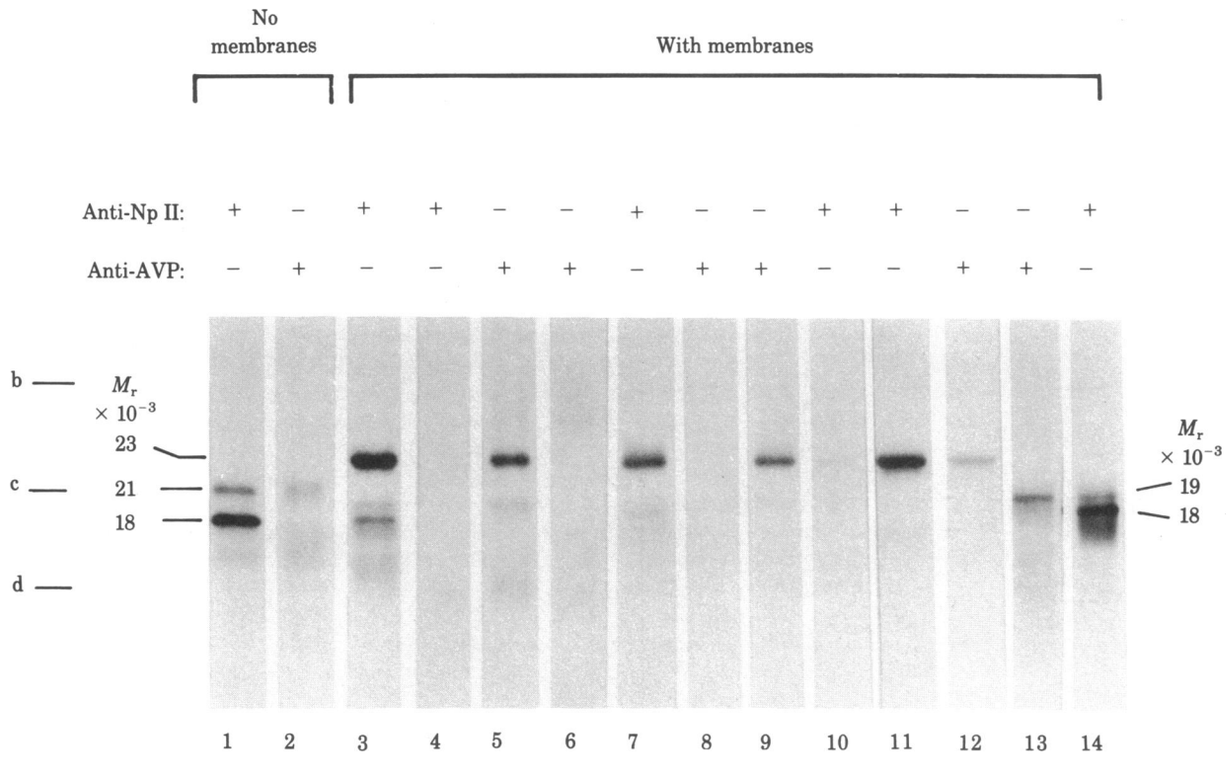

FIG. 2. Synthesis and processing of the Np II-AVP precursor in the absence and presence of microsomal membranes and sequential immunoprecipitation. Translation of bovine hypothalamic mRNA in the reticulocyte lysate system, immunoprecipitation, and gel electrophoresis were carried out as described in Fig. 1. Lanes 3–12: 5 A_{260} units of dog pancreas microsomal membranes per ml of assay mixture. Lanes 13 and 14: 3 A_{260} units of microsomal membranes from tunicamycin-treated ascites tumor cells (15) were added per ml of assay mixture prior to incubation. Lanes 4 and 6: immunoprecipitation in the presence of excess Np II or AVP, respectively (10 μg). Lanes 7–10 sequential immunoprecipitation: 1st step, anti-Np II (lane 7); 2nd step, anti-AVP (lane 8); 1st step, anti-AVP (lane 9); 2nd step, anti-Np II (lane 10). Lanes 11 and 12: Np II-AVP precursor synthesized in the presence of dog pancreas microsomal membranes, immunoprecipitated with anti-Np II (lane 11) or anti-AVP (lane 12), bound to concanavalin A-Sepharose columns and eluted with 0.2 M α-methyl mannoside (24). The M_r 19,000 and 18,000 products were not retained on the columns and appeared in the sugar-free eluate (data not shown). The M_r 23,000 product was bound to the column quantitatively and more than 80% was recovered after elution with α-methyl mannoside. For M_r markers, see Fig. 1.

iments, in particular when the first immunoprecipitation was with anti-AVP, the second step yielded minor immunoreactive material with anti-Np II (see also Fig. 2, lanes 9 and 10). Apparently, this result was due to an excess of the M_r 21,000 product over anti-AVP and could be overcome by using higher concentrations of anti-AVP.

Processing and Core Glycosylation of the Precursor. The existence of a common precursor to neurophysin II and to the oligopeptide hormone AVP suggests that the M_r 21,000 precursor contains a signal or pre sequence at the NH_2 terminus as has been shown for other polypeptide hormones (20–23). In general, the pre sequence, consisting of 20–30 amino acids, is cleaved off cotranslationally by proteolytic enzyme(s), to yield a pro form slightly lower in molecular weight than the prepro form.

When hypothalamic mRNA was translated in the presence of a microsomal membrane fraction from dog pancreas, the M_r 21,000 precursor was converted to a minor M_r 19,000 and a prominent M_r 23,000 product identified by anti-Np II (Fig. 2, lane 3) or anti-AVP (Fig. 2, lane 5). That both the M_r 19,000 and 23,000 polypeptides are common precursors to Np II and AVP was again demonstrated by sequential immunoprecipitation (Fig. 2, lanes 7–10). The recent suggestions (13) that the M_r 18,000 polypeptide might also be processed to a lower molecular weight product was most likely due to the less-specific antibodies used in those experiments, which were unable to discriminate between Np I and Np II.

The M_r 23,000 product could be identified as the glycosylated pro form in two ways. First, the M_r 23,000 polypeptide, but neither the 19,000 nor the 21,000 polypeptide, was retained on a concanavalin A-Sepharose affinity column. The core-glycosylated M_r 23,000 product could be eluted from the column with 0.2 M α-methyl mannoside (Fig. 2, lanes 11 and 12). Second, when translation of hypothalamic mRNA was performed in the presence of a microsomal membrane fraction derived from tunicamycin-treated ascites tumor cells, the M_r 19,000 pro form (lanes 13 and 14) was synthesized but its core-glycosylated M_r 23,000 polypeptide was not. The antibiotic tunicamycin is known to block *in vivo* formation of the activated carbohydrate-lipid intermediates that are essential prerequisites in the membrane-dependent glycosylation of glycopolypeptides (25); processing of the prepro form to the pro form, however, is unaffected (15).

DISCUSSION

The data presented here show cell-free ribosome-dependent synthesis of a polypeptide precursor containing an amino acid sequence immunologically identical to that of the nonapeptide hormone AVP. Sequential immunoprecipitation studies indicate that this oligopeptide exists together with its carrier protein Np II as a composite common precursor, supporting recent *in vivo* studies (7, 18). That the two peptides are part of a common precursor was also supported by the finding that with both anti-AVP and anti-Np II, electrophoretically identical pro forms,

either glycosylated or nonglycosylated, could be obtained. Our data show a M_r 21,000 prepropolypeptide which can be converted into a M_r 19,000 pro form, implying a pre sequence of approximately 15 amino acids. The latter can also be core glycosylated to a M_r 23,000 product. With anti-Np II but not with anti-AVP, another precursor polypeptide (M_r 18,000) could be identified that differs from the M_r 21,000 product by approximately 30 amino acids. Incorporation studies with *N*-formyl[^{35}S]methionine suggest that the NH_2 terminus of the M_r 21,000 and the M_r 18,000 polypeptide is intact (13); consequently, both precursors should be prepro forms. Provided that the two polypeptides are synthesized from one mRNA, the 30 amino acids that lack the M_r 18,000 polypeptide should be located at the COOH-terminus of the M_r 21,000 precursor. If this assumption is correct, the AVP sequence should be at or near the COOH-terminus of the M_r 21,000 prepro form. On the other hand, if the M_r 18,000 polypeptide represents another discrete Np II-like precursor (18), then the AVP sequence would not necessarily be at the M_r 21,000 COOH-terminus. The precise arrangements of the two polypeptides Np II and AVP within the precursor will have to await amino acid sequence analysis.

The data presented here are in good agreement with those obtained by direct extraction of neurophysin-like peptides from neurosecretory granules of the neurohypophysis (9, 18). In those experiments, larger precursors (presumably pro forms) having immunoreactive sequences corresponding to neurophysins as well as to vasopressin were reported with M_r of 20,000–30,000. Recently, a M_r 40,000 potential precursor isolated under denaturing conditions was described (26). To date we have no evidence to suggest the existence of such a translation product arising from bovine hypothalamic mRNA. Even when the translation assay was supplemented with total calf liver tRNA, including termination suppressor species in order to encourage read-through of potentially long mRNA, no high molecular weight products were observed (unpublished data).

The existence of composite common precursors has been demonstrated for other neuropolypeptides of which the best studied example is pro-opiocortin (27) or pro-opiomelanocortin (28) containing corticotropin, β-lipotropin, endorphins, and melanotropins (27–32). A common precursor has also been described which includes seven copies of [Met]enkephalin and one [Leu]enkephalin (33). These precursors from mammmalian tissue bear a close resemblance to viral polyproteins in that both exhibit the unusual property of comprising more than one functionally distinct polypeptide (34). In both cases a central role is played by the endoplasmic reticulum which appears to define a topology for the various processing and modification events. Although similar in many ways, to date only the viral polyproteins offer evidence of cotranslational proteolysis of the nascent translation product by viral as well as cellular proteases (35).

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