IDENTIFICATION AND PROCESSING OF BIOSYNTHETIC PRECURSORS TO VASOACTIVE INTESTINAL POLYPEPTIDE IN HUMAN NEUROBLASTOMA CELLS

Ken-ichi OBATA, Nobuyuki ITOH, Hiroshi OKAMOTO*, Chizuko YANAIHARA+, Noboru YANAIHARA+ and Toshimitsu SUZUKI†

Department of Biochemistry, Toyama Medical and Pharmaceutical University School of Medicine, Toyama 930-01, +Laboratory of Bioorganic Chemistry, Shizuoka College of Pharmacy, Shizuoka 422 and *†Department of Pathology, Niigata University School of Medicine, Niigata 951, Japan*

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1. Introduction

Vasoactive intestinal polypeptide (VIP), a 28-amino acid peptide, was originally isolated from porcine duodenum [1]. VIP is present not only in gastrointestinal tissues but also neural tissues [2,3]. Therefore, VIP may act as a neurotransmitter [3]. VIP has also been found in neuroblastoma cell lines [4–6]. The biosynthetic mechanism of VIP, however, remains to be elucidated.

Here, we present evidence indicating that VIP is synthesized from a precursor (pro-VIP) of estimated M_r 17 500, in human neuroblastoma NB-I cells. By the translation of poly(A)-containing RNA prepared from NB-I cells in a cell-free translation system of rabbit reticulocyte lysate, we have also identified a primary translation product of VIP mRNA (prepro-VIP) of $M_r \sim 20\,000$. In addition, we have found that the synthesis of 'pro-VIP' in NB-I cells is stimulated by dibutyryl cAMP.

2. Experimental

2.1. *Materials*

L-[^{35}S]Methionine (1300–1400 Ci/mmol) was purchased from Amersham; fetal calf serum from Gibco; goat antiserum to rabbit IgG from Miles-Yeda; Trasylol from Bayer; M_r-marker proteins (M_r-marker) from Oriental (Osaka). Rabbit antiserum to synthetic porcine VIP was produced as in [7].

* To whom correspondence should be addressed

2.2. *Cell culture*

Human neuroblastoma cell line NB-I was originally established in [8]. The cells were grown in RPMI 1640 containing 10% fetal calf serum and 0.5 mg kanamycin/ml.

2.3. *Labeling NB-I cells with [^{35}S]methionine*

NB-I cells, grown in the above medium, were washed twice with Hanks solution and incubated at 37°C for 3 h in Dulbecco's modified Eagle medium (without methionine) containing 10% fetal calf serum, 0 or 1 mM dibutyryl cAMP and 500 μCi [^{35}S]methionine/ml.

2.4. *Preparation of poly(A)-containing RNA*

Poly(A)-containing RNA was prepared from dibutyryl cAMP-induced NB-I cells by phenol extraction followed by LiCl-precipitation and oligo(dT)-cellulose chromatography as in [9,10].

2.5. *Cell free translation*

Poly(A)-containing RNA was translated at 25°C for 4 h in a cell-free system of rabbit reticulocyte lysate [11] containing 500 μCi [^{35}S]methionine/ml. After incubation, the reaction was stopped by the addition of 0.4 vol. 1 mg RNase A/ml followed by incubation at 37°C for 20 min.

2.6. *Immunoprecipitation procedure*

The NB-I cells labeled with [^{35}S]methionine were sonicated in 1 ml 0.1% SDS buffer (0.1% SDS, 0.1% Triton X-100, 100 unit/ml Trasylol, 10 mM Tris–HCl, pH 7.4, 2 mM EDTA) containing 0.1 vol. rabbit reticulocyte lysate, heated at 100°C for 1 min, and cen-

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trifuged at 10 000 rev./min for 20 min at 2°C after adding 0.1 vol. 1.4 M NaCl. To aliquots of the cell-extracts were added 1 μl antiserum to VIP and 0 or 1 μg VIP, and the solutions were incubated at 4°C for 48 h. After incubation, 10 μl antiserum to rabbit IgG was added to each extract followed by further incubation at 4°C for 16 h. Resultant immunoprecipitates were collected by centrifugation at 10 000 rev./min for 60 min at 2°C through 1 ml 1 M sucrose in 0.1% SDS buffer containing 0.14 M NaCl. The immunoprecipitates were then successively washed with 0.1 M sodium acetate (pH 4.6), 0.1 M sodium borate (pH 8.8) and 0.1% SDS buffer essentially as in [12]. In cell-free translation assays, the translation mixture was diluted with 10 vol. 0.1% SDS buffer, heated at 100°C for 1 min and centrifuged at 10 000 rev./min for 20 min at 2°C after adding 0.1 vol. 1.4 M NaCl and 0.01 vol. 20 mM unlabeled methionine. The immunoprecipitation procedures were the same as above.

2.7. *SDS–Polyacrylamide gel electrophoresis*

The immunoprecipitates were dissolved in a sample buffer (60 mM Tris–phosphoric acid, pH 6.8, 1% SDS, 10% 2-mercaptoethanol, 10% glycerol) containing M_r-marker proteins and analyzed by SDS-polyacrylamide (15%) gel electrophoresis. After electrophoresis, the ^{35}S-labeled products were detected by autoradiofluorography [13].

3. Results and discussion

NB-I cells were cultured with [^{35}S]methionine at 37°C for 3 h. The cell-extract was immunoprecipitated with antiserum to VIP in the presence or absence of unlabeled synthetic VIP and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiofluorography. The results are shown in fig.1(a,b). Although several products labeled with [^{35}S]methionine were detected, only 2 products, one product at the position corresponding to VIP and another product of estimated M_r 17 500, were effectively eliminated by an excess of unlabeled VIP during the immunoprecipitation reaction. These results strongly suggested that the 2 products reacted specifically with the antiserum. In a chase experiment, the [^{35}S]methionine-labeled NB-I cells were further incubated at 37°C for 3 h after adding unlabeled methionine. The cell-extracts were immunoprecipitated and analyzed by SDS–polyacrylamide electrophoresis followed by autoradiofluorography. Although the

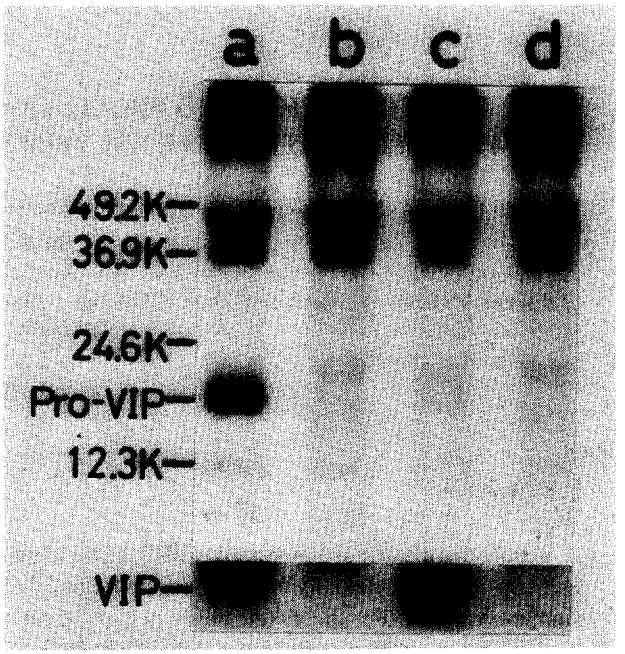

Fig.1. Autoradiofluorogram of SDS–polyacrylamide gel of [^{35}S]methionine-labeled NB-I cell-extract immunoprecipitates. NB-I cells were pulse-labeled with [^{35}S]methionine at 37°C for 3 h. Immunoprecipitates were prepared from the cell-extracts with antiserum to VIP in the presence or absence of unlabeled VIP and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiofluorography: (a) no VIP; (b) 1 μg VIP. The ^{35}S-labeled NB-I cells were further incubated at 37°C for 3 h after adding unlabeled methionine. Immunoprecipitates were prepared from the cell-extracts and analyzed as above: (c) no VIP; (d) 1 μg VIP. The product corresponding to VIP was detected with the prolongation of exposure-time in autoradiofluorography, as VIP contained only 1 methionine residue [1].

labeled 17 500-M_r product decreased in the chase experiment, the labeled product at the position corresponding to VIP increased (fig.1(c,d)). These results indicated that the 17 500-M_r product was a precursor to VIP (pro-VIP).

Dibutyryl-cAMP was known to induce some neural specific enzymes, such as tyrosine hydroxylase, choline acetyltransferase and acetylcholine esterase, as well as the morphological differentiation in neuroblastoma cells [14]. VIP-like immunoreactive materials in NB-I cells were also increased by dibutyryl-cAMP [15]. Here, we examined the effect of dibutyryl-cAMP on the synthesis of pro-VIP in NB-I cells. Although the synthesis of total protein was essentially unchanged by dibutyryl-cAMP, the synthesis of pro-VIP was stimulated ~2-fold (table 1).

Table 1
Effect of dibutyryl cAMP on synthesis of total protein and pro-VIP in NB-I cells ([^{35}S] cpm/2 × 10^5 cells)

Dibutyryl cAMP	Total protein synthesis	Pro-VIP synthesis
Exp. 1		
0 mM	1.38 × 10^6 (1.00)	602 (1.00)
1 mM	1.67 × 10^6 (1.21)	1358 (2.25)
Exp. 2		
0 mM	1.30 × 10^6 (1.00)	609 (1.00)
1 mM	1.44 × 10^6 (1.11)	1477 (2.42)

NB-I cells grown for 48 h in the presence or absence of 1 mM dibutyryl-cAMP were incubated at 37°C for 3 h with [^{35}S]-methionine in the presence or absence of dibutyryl cAMP. Total protein synthesis was determined with aliquots of the cell extracts. For determination of pro-VIP synthesis, immunoprecipitates were prepared with antiserum to VIP and analyzed by SDS–polyacrylamide gel as in fig.1. The bands corresponding to pro-VIP were excised from the gels to determine the radioactivity of pro-VIP. The numbers in parentheses were related to that incubated without dibutyryl cAMP

To identify a primary translation product of VIP mRNA, poly(A)-containing RNA prepared from NB-I cells was translated in a cell-free system of rabbit reticulocyte lysate containing [^{35}S]methionine. The translation products were analyzed by immunoprecipitation and SDS–polyacrylamide gel electrophoresis followed by autoradiofluorography. The result is shown in fig.2. Only one labeled product, which reacted specifically with antiserum to VIP, was detected (fig.(a,b)). The product, which migrated a little slower than pro-VIP (fig.(c,d)) was of estimated M_r 20 000. Although the amino acid sequence of the primary translation product is not yet elucidated, we estimated that the primary translation product, prepro-VIP, consisted of the NH_2-terminal pre-peptide and pro-VIP as reported in the case of other peptide hormones.

Other neuropeptide-like immunoreactivity, such as substance P-, neurotensin- and somatostatin-like immunoreactivity, was also detected in NB-I cells [6]. As VIP was shown to have M_r 3500 (28 amino acid residues) [1], the size of pro-VIP was ~5-times that of VIP. Therefore, other neuropeptides may be contained in the pro-VIP structure. To resolve this problem, we are now attempting the construction and molecular cloning of a recombinant DNA containing VIP cDNA to elucidate the structure of (pre)pro-VIP.

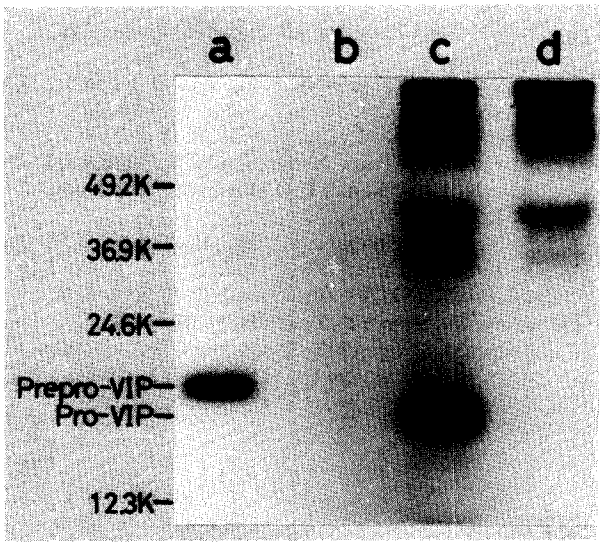

Fig.2. Autoradiofluorography of SDS–polyacrylamide gel of [^{35}S]methionine-labeled immunoprecipitated cell-free translation products of poly(A)-containing RNA from NB-I cells. Poly(A)-containing RNA was prepared from NB-I cells grown for 48 h in the presence of 1 mM dibutyryl-cAMP, and translated in a cell-free translation system of rabbit reticulocyte using [^{35}S]methionine. Immunoprecipitates were prepared from the cell-free translation products with antiserum to VIP in the presence or absence of VIP and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiofluorography: (a) no VIP; (b) 1 μg VIP. In a parallel experiment, the NB-I cells were also incubated at 37°C for 3 h in the presence of [^{35}S]methionine and 1 mM dibutyryl-cAMP. Immunoprecipitates were prepared and analyzed as above: (c) no VIP; (d) 1 μg VIP.

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