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CELL-FREE TRANSLATION OF HUMAN PHEOCHROMOCYTOMA MESSENGER RNA YIELDS PROTEIN(S) CONTAINING METHIONINE-ENKEPHALIN

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ABSTRACT

Cell-free translation of messenger RNA extracted from a human pheochromocytoma yields protein(s) of apparent molecular weight >70,000 daltons which contain the pentapeptide methionine-enkephalin. It is estimated that 0.8-1.0% of the total pheochromocytoma mRNA codes for the methionine-enkephalin-containing protein, based on percent incorporation of [^{35}S]methionine into methionine-enkephalin during cell-free translation. These results demonstrate that human pheochromocytoma mRNA contains the message for a high-molecular weight methionine-enkephalin-containing protein or proteins, presumably the methionine-enkephalin precursor molecule(s).

INTRODUCTION

Adrenal medullas of several species including man contain substantial quantities of enkephalin and putative enkephalin precursors (1-5). Human pheochromocytomas are very rich in enkephalin and related peptides (6-9). Methionine-enkephalin and leucine-enkephalin appear to be synthesized in cow and human adrenal as high-molecular weight precursor(s) containing multiple copies of the enkephalin pentapeptide sequences (4,5,8,10,11). An understanding of the biosynthesis of enkephalin in the adrenal medulla depends on identification of and characterization of the initial polypeptide product translated from the messenger RNA transcribed from the "enkephalin" cistron.

ABBREVIATIONS: ME, methionine-enkephalin; LE, leucine-enkephalin; RP-HPLC, reverse-phase high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; TPCK, L - (tosylamido 2-phenyl) ethyl chloromethylketone.

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We report that ME can be generated from protein products of cell-free translation of pheochromocytoma mRNA following enzymatic digestion, that the apparent molecular weight of these protein product(s) is greater than 70,000 daltons, and that the mRNA which codes for ME-containing protein constitutes a significant fraction of the total pheochromocytoma mRNA.

METHODS

Isolation of RNA: Four gm of human pheochromocytoma was homogenized in a Teflon-glass homogenizer in 4 volumes of 3M LiCl, 4M urea, 0.01 M Na acetate pH 5.0 containing 100 µg/ml heparin and 0.1% SDS, filtered through sterile gauze and kept at 0°C overnight. The homogenate was centrifuged at 40,000 x g for 15 min and the pellet dissolved in 1 vol of 6 M LiCl, 8 M urea, 0.01 M Na acetate and 0.02 M EDTA. This solution was extracted once with 2 vol of chloroform - phenol (1:1, v/v) and twice with 1.2 vol of chloroform - phenol. The aqueous phase was diluted with 3 vol of absolute ethanol and placed at -20°C overnight to allow precipitation of RNA, which was collected by centrifugation at 5000 x g for 30 min. RNA was dissolved in water and stored at -70°C.

Cell-free Translation: Aliquots corresponding to 0.5-1.0 mg wet weight of tissue extracted were translated in the presence of [^{35}S] methionine in a rabbit reticulocyte lysate system (New England Nuclear) prepared after the method of Pelham and Jackson (12). After one hour of incubation at 35°C, the translation was terminated by the addition of 1.2 µg of RNase A and EDTA and diluted with 3.8 vols (300 µl) of 0.01 M Tris HCl, pH 7.5, containing 0.1 M NaCl, 0.001 M EDTA, 0.1% SDS, 0.5% deoxycholate and 1% Triton X-100. Samples were applied to a Sephadex G-25 column and eluted with 0.05 M Tris pH 8.0. The peak of radioactive material eluting prior to the elution of [^{35}S]-methionine was lyophilized.

Enzymatic Digestion of Translation Products: Radiolabeled material eluted from the G-25 column was suspended in 0.1 M Tris-HCl, pH 8.0, containing 100 pg/ml of unlabeled ME. Samples (1 ml total volume) were treated for 20 min at 37°C with TPCK-treated trypsin (Worthington; 25 µg/ml), incubated at 95°C for 10 min to inactive trypsin, incubated at 37°C for 20 min with carboxypeptidase B (0.1 µg/ml) and again incubated at 95°C for 10 min to inactivate carboxypeptidase B.

Immunoprecipitation of ME: Following enzymatic digestion, the mixture was divided into two aliquots of 500 µl each. To one aliquot was added 50 µg of unlabeled ME and to both 10 µl of rabbit anti-ME antiserum (antiserum RB-4, a kind gift of Dr. S. Sabol). Tubes were incubated overnight at 4°C and 50 µl of anti-rabbit goat gammaglobulin (Calbiochem) was added to precipitate the immune complex. Tubes were centrifuged after 12 hr at 4°C and the pellet rinsed once with incubation buffer and dissolved in 700 µl of 3% beta-mercaptoethanol by incubation at 37°C for 2 hours and boiling for 30 min.

High pressure Liquid Chromatography: Samples were chromatographed by reverse-phase HPLC employing a 4.6 x 250 mm Supelco Supelcosil 5 µ LC-18 column and eluted with a mobile phase consisting in triethylammonium formate, 0.25 M pH 3.0 and acetonitrile according to the following solvent program: 5 min isocratic 15% acetonitrile followed by a 5 min linear gradient to 20% acetonitrile, 5 min of isocratic elution at 20% acetonitrile, and finally a linear gradient over 10 min to 60% acetonitrile.

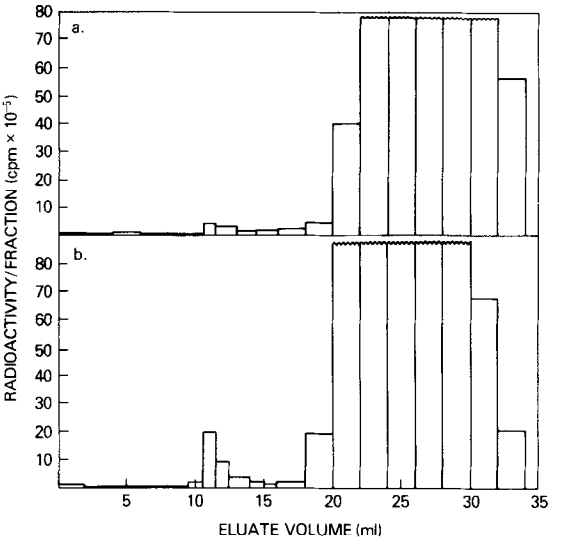

Fig. 1. G-25 chromatography of [^{35}S]labeled translation products. Following cell-free translation of pheochromocytoma mRNA in the presence of [^{35}S]-methionine, samples were applied to a Sephadex G-25 Superfine column (18 x 170 mm) and eluted with 0.05 M Tris pH 8.0. Radioactivity in aliquots of each fraction was measured by liquid scintillation spectrometry. Material eluting from the column between 10 and 15 ml was pooled and submitted to enzymatic digestion and immunoprecipitation (Fig. 2). a. Radioactive elution profile in the absence of pheochromocytoma RNA added to the translation cocktail. b. Radioactive elution profile in the presence of pheochromocytoma RNA added to the translation cocktail.

RESULTS AND DISCUSSION

RNA was extracted as described in Methods from 4 gm of human pheochromocytoma obtained and frozen shortly after tumor resection. Messenger RNA was translated in vitro using the rabbit reticulocyte system and the [^{35}S]-labeled translation products were separated from [^{35}S]methionine by Sephadex G-25 chromatography (Fig. 1) and submitted to sequential trypsin and carboxypeptidase B digestion. These enzymatic treatments have been shown to liberate ME and LE from high-molecular weight polypeptides of both cow adrenal medulla and human pheochromocytoma (4,9). Enzymatic digests were immunoprecipitated after incubation with anti-ME antiserum, and the immunoprecipitated products chromatographed by reverse-phase high performance liquid chromatography. The majority of the [^{35}S]-labeled material immunoprecipitated with

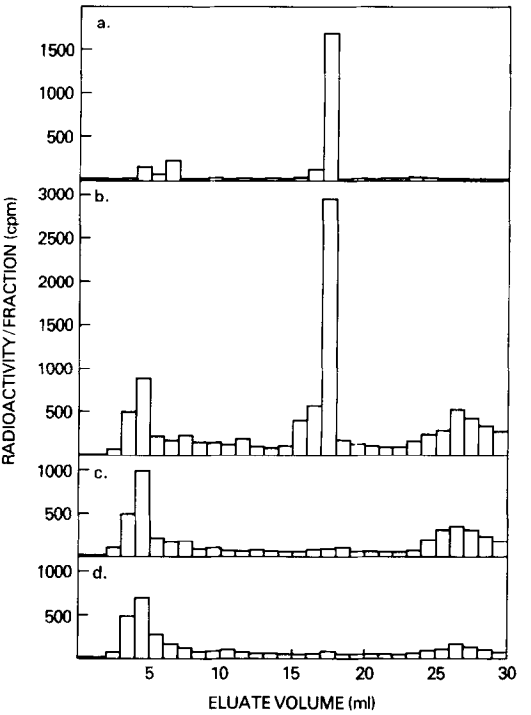

Fig. 2. RP-HPLC chromatography of enzyme-digested translation products immunoprecipitated with anti-ME antiserum. Material eluting from the G-25 Sephadex column at 10-15 ml was pooled, lyophilized, submitted to trypsin and carboxypeptidase B digestion and immunoprecipitated as described in Methods. The immunoprecipitate was dissolved in 3% β-mercaptoethanol, and brought to 0.25 M triethylammonium formate pH 3.0, in a volume of 1 ml. Samples were chromatographed by reverse-phase HPLC as described in Methods and 1 ml fractions measured for radioactivity of liquid scintillation spectrometry. Authentic ME has a retention time of 17-18 ml in this system. Samples containing authentic [^{3}H]ME were also taken through the enzymatic digestion, immunoprecipitation, and HPLC steps described above. Recovery of authentic [^{3}H]ME (Amersham) was 30%. a. Authentic [^{3}H]ME carried through enzymatic digestion, immunoprecipitation and HPLC. b. Pheochromocytoma mRNA translation products following enzymatic digestion and immunoprecipitation. c. As in b. except that the immunoprecipitation was carried out in the presence of excess (50 μg) ME. d. as in b. except that translation was carried out in the absence of added pheochromocytoma mRNA.

anti-ME antiserum co-chromatographed with authentic ME in the reverse-phase HPLC system employed (Fig. 2).

In order to estimate the molecular weight of the translation products containing the ME sequence, the [^{35}S]products eluting in the void volume after

Sephadex G-25 chromatography were re-chromatographed on Sephadex G-200 and column fractions were assayed for [^{35}S]ME by enzymatic digestion and immunoprecipitation as described above. [^{35}S]ME was only generated by digestion of fractions 5 and 6 (elution volume 4.3-6.9 ml). As shown in Figure 3, proteins that comprise these fractions have apparent molecular weights of 70-180,000 daltons. Previous investigations carried out in bovine chromaffin cells and bovine adrenal medulla have demonstrated the presence of an approximately 50,000 dalton enkephalin-containing protein and a precursor-product relationship between high-molecular weight ME-containing proteins and ME itself (4,13). The present data confirm these observations and suggest that if the enkephalin biosynthetic pathway in pheochromocytoma is similar to that in the bovine adrenal medulla, the 50,000 dalton putative precursor is itself a product of the processing of an even larger precursor. In addition, our failure to detect lower-molecular weight ME-containing proteins implies that the 14,000 dalton ME-containing protein of the human pheochromocytoma (9) and the multiple ME-containing proteins of the bovine adrenal medulla which are less than 50,000 daltons may also derive from a precursor which is at least greater than 70,000 daltons.

The percent of pheochromocytoma message coding for the ME-containing protein(s) was estimated by determining the amount of [^{35}S]ME relative to total [^{35}S]-labeled translation products. 0.8-1.1% of the total [^{35}S]-label appears in [^{35}S]ME (Table 1). Thus, messenger RNA coding for the ME-containing protein(s) constitutes a significant proportion of the total mRNA of human pheochromocytoma.

The present results demonstrate the cell-free translation of ME-containing high-molecular weight protein, or putative ME precursor. It appears that the human pheochromocytoma is a rich source of messenger RNA coding for the ME precursor.

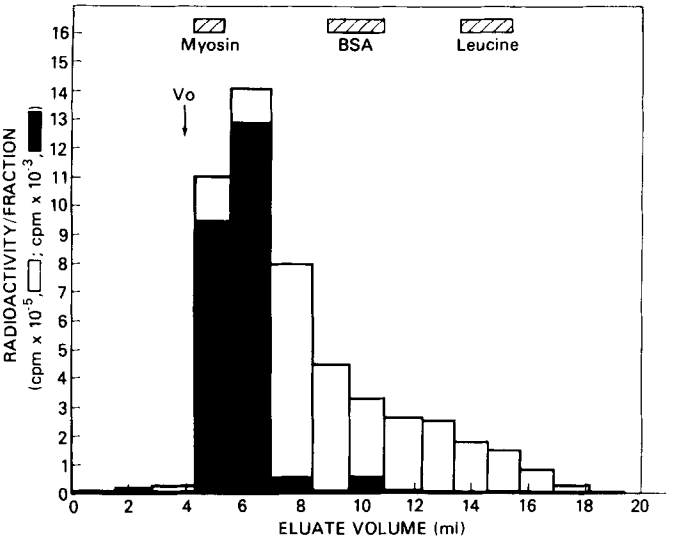

Fig. 3. Sephadex G-200 chromatography of [^{35}S]labeled translation products. [^{35}S]labeled translation products were chromatographed on Sephadex G-25 and radioactive material eluting between 10 and 15 ml collected and lyophilized. Samples were resuspended in 1 ml of 50 mM ammonium acetate and chromatographed on a Sephadex G-200 column (5 x 70 mm) eluted with 50 mM ammonium acetate. 25-Drop fractions were collected, lyophilized, resuspended in Tris buffer and submitted to enzymatic digestion and anti-ME immunoprecipitation as described in Methods. Elution volume is plotted against radioactivity eluted per fraction (□) and [^{35}S]labeled material specifically immunoprecipitated with anti-ME after enzymatic digestion (■). Molecular weight markers are [^{14}C]methylated myosin (Amersham) 200,000 daltons, bovine serum albumin (Pharmacia) 69,000 daltons, and [^{3}H]leucine (Amersham). V_0 indicates the elution volume of dextran blue.

Table 1 Estimate of percentage of pheochromocytoma mRNA coding for ME-containing protein(s)

Experiment No.	Total Translated Products, cpm	[^{35}S]ME, cpm	% Total ^{35}S in [^{35}S]ME
1	3,000,000	24,352	0.812%
2	2,086,787	23,577	1.13%
3	2,458,420	19,827	0.806%

Total translated products represents cpm ^{35}S eluted from the G-25 column (10-15 ml) following translation. Background (cpm ^{35}S eluted from the G-25 column after translation in the absence of added mRNA) has been subtracted from each sample and was always less than 800,000 for any translation. [^{35}S]-ME was calculated as cpm appearing at the retention time of authentic ME during RP-HPLC following translation, enzymatic digestion, and immunoprecipitation (see Figure 2) and corrected for recovery using the recovery of [^{3}H]ME taken through the above procedures (average recovery of [^{3}H]ME 30%).

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