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Studies with [^{35}S]methionine indicate that the 22,000-dalton [Met]enkephalin-containing protein in chromaffin cells is a precursor of [Met]enkephalin

(immunoprecipitation/adrenal/prohormone/cell culture)

JEAN ROSSIER*, JOSÉ M. TRIFARÓ†, RANDOLPH V. LEWIS‡, RAYMOND W. H. LEE†, ALVIN STERN§, SADAO KIMURA¶, STANLEY STEIN§, AND SIDNEY UDENFRIEND§

*Physiologie nerveuse, Centre National de la Recherche Scientifique, 91190-Gif-sur-Yvette, France; †Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada H3G 1Y6; ‡Department of Biochemistry, University of Wyoming, Laramie, Wyoming 82071; §Roche Institute of Molecular Biology, Nutley, New Jersey 07110; and ¶Tsukuba University, Niihari, Ibaraki, Japan

Contributed by Sidney Udenfriend, July 21, 1980

ABSTRACT **It has been shown that [^{35}S]methionine is incorporated into the [Met]enkephalin sequences of a 22,000-dalton enkephalin-containing protein in the adrenal medulla. Pulse–chase experiments indicate that label is incorporated into the large polypeptide before it appears in free [Met]enkephalin and a smaller [Met]enkephalin-containing peptide. These findings provide direct evidence of a precursor–product relationship of these structurally related polypeptides.**

The bovine adrenal medulla contains several large polypeptides, ranging in size from >50,000 to 1000 daltons, that contain enkephalin sequences but are not related to β-endorphin. Many of these polypeptides have been purified to homogeneity and the sequences of some have been partially determined (1–3). Each of these polypeptides contains one or more copies of enkephalin, which is indicative of their role as precursors and intermediates in the synthesis of the opioid pentapeptides. Previous experiments had shown that bovine chromaffin cells cultured for up to 10 days maintain constant levels of enkephalin and enkephalin precursors (4). This suggests that these cells in culture actively synthesize the enkephalins and their precursors. To establish the precursor relationship of these large molecules, pulse-labeling experiments were carried out. It was shown that, after a 2-hr pulse, radioactive methionine was incorporated into one of the large enkephalin precursors (22,000 daltons) with no measurable incorporation into free [Met]-enkephalin. After an additional 6 hr of incubation after the pulse, radioactivity was also found in [Met]enkephalin and in the recently characterized enkephalin congener, [Met]enkephalin-Arg^6,Phe^7 (5).

MATERIALS AND METHODS

Isolation and Culture Cells. Chromaffin cells were isolated from bovine adrenal glands and purified by a modification (6) of the method of Livett *et al.* (7). The cells were maintained in culture by plating onto collagen-coated plastic dishes under conditions as described (6, 7).

Labeling with [^{35}S]Methionine. Twelve plates (10^6 cells per plate) of 8-day-old cells were washed once with a modified Dulbecco's modified Eagle's medium (DME medium) containing 0.01% of the normal DME medium concentration of amino acids and supplemented with fetal calf serum (10%), ascorbic acid (0.1 mM), and Hepes (15 mM). The plates were incubated in this modified DME medium for 2 hr in the presence of 22.5 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine per plate (1046 Ci/mmol; New England Nuclear). Labeled medium was then replaced with the modified DME medium and incubation was continued for an additional 5 min at 37°C. The plates were washed three more times and the washed cells from six plates were scraped off with 1 M acetic acid and lysed by two cycles of freezing and thawing. The lysed cells were centrifuged at 105,000 × *g* for 1 hr and the supernatant was lyophilized. After the 2-hr pulse, the six remaining plates were incubated with amino acid-supplemented DME medium for an additional 6 hr. The cells were then collected and lysed as described above.

Immunoprecipitation. Antiserum JR 235 prepared against the NH_2 terminus of [Met]enkephalin was used (3). At a final dilution of 1:600 under the conditions of our radioimmunoassay this antiserum crossreacted with [Met]enkephalin-Lys^6 (42%), [Met]enkephalin-Arg^6 (19%), [Met]enkephalin-Arg^6,Phe^7 (23%), and the sulfoxide of [Met]enkephalin (100%). For immunoprecipitation, 10 μl of this antiserum was incubated with each sample for 2 days at 4°C with 40 μl of 10 mM sodium phosphate, pH 7.4/145 mM NaCl/0.01% Thimerosal/0.1% gelatin/0.01% crystalline bovine serum albumin/0.1% Triton X-100. The second precipitation was achieved with 1 ml of a *Staphylococcus aureus* protein A preparation (IgSorb, Enzyme Center, Boston, MA) diluted 1:4 in the medium described above. After a 10-min incubation at 20°C and a 2-min centrifugation at 10,000 × *g*, the supernatant was discarded and the pellet was assayed for radioactivity. Blanks were obtained by adding 1 μg of nonradioactive [Met]enkephalin to each extract to displace radioactive antigen in them. Column fractions were evaporated to dryness and redissolved in 25 μl of 50% ethanol in water prior to immunoprecipitation.

RESULTS

Incorporation into the 22,000-Dalton Protein. After incubation of the cells with [^{35}S]methionine, the cellular extract was treated with trichloroacetic acid to precipitate proteins. The supernatant solution containing enkephalins, hexapeptides, and heptapeptides (equivalent to fraction V in ref. 1) was set aside (see below). The precipitate, containing proteins and large polypeptides, was redissolved in 4 M urea and subjected to gel filtration on a Sephadex G-75 column, and the fractions corresponding to the 22,000-dalton putative enkephalin precursor (fraction I, tubes 10–12) were pooled (4). The pooled fractions were evaporated to dryness and redissolved in 1 ml of 50 mM Tris·HCl (pH 8.0) containing 100 μg of TPCK-treated trypsin

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Abbreviations: DME medium, Dulbecco's modified Eagle's medium; HPLC, high-pressure liquid chromatography.

Table 1. Radioactivity in tryptic peptides of the 22,000-dalton protein

Tubes	Specific immunoprecipitate	Blank	Difference (enkephalin-containing peptides)	Elution position of
15	130	95	35	
16	200	75	125	
17	695	175	520	[Met]enkephalin-Lys^6
18	765	190	575	[Met]enkephalin-Arg^6
19	135	105	30	
20	160	80	80	
21	165	110	55	[Met]enkephalin
22	90	30	60	

Fractions 15–22 obtained by HPLC were evaporated to dryness and resuspended in 25 μl of 50% ethanol in water. An aliquot (5 μl) was precipitated with specific antiserum. Blanks were obtained by adding 1 μg of nonradioactive [Met]enkephalin to a second 5-μl aliquot to displace specifically radioactive antigenic material in the mixture. Values represent counts above background and were multiplied by 5 to give value for entire fraction.

(Worthington). After 16 hr at 37°C, the trypsin digests were separated by high-pressure liquid chromatography (HPLC). As expected, radioactivity was found in many peptide peaks. However, from previous studies (1) it was known that incubation of the 22,000-dalton putative precursor with trypsin yields [Met]enkephalin-Lys^6, [Met]enkephalin-Arg^6, and [Met]enkephalin. Accordingly, tubes 15–22 from the HPLC, corresponding to the markers for these peptides, were collected and 5-μl aliquots of each tube were subjected to immunoprecipitation, as described above. The radioactivity in the specific immunoprecipitates was appreciable in tubes 17 and 18 corresponding to [Met]enkephalin-Lys^6 and [Met]enkephalin-Arg^6. (Table 1). Little radioactivity appeared in the immunoprecipitate in fraction 21 corresponding to [Met]enkephalin.

The immunoprecipitates obtained with fractions 17 and 18 accounted for about 6% of the radioactivity in these fractions. The antiserum precipitates at best 40% of pure enkephalins when they are present in trace amounts. By correcting for recoveries during HPLC and immunoprecipitation, it can be estimated that at least 13% of the radioactivity in fractions 17 and 18 was incorporated into [Met]enkephalin-Lys^6 (or)-Arg^6 or both. On the basis of this value, 13%, at least 0.8% of the radioactivity in the crude fraction I is associated with enkephalin sequences found in the 22,000-dalton enkephalin precursor.

Incorporation into Fraction V (Trichloroacetic Acid Extract). When fraction V from the 2-hr pulse was analyzed by HPLC, no radioactivity was found associated with [Met]enkephalin or [Met]enkephalin-Arg^6,Phe^7 (Table 2). Results of the 2-hr pulse and 6-hr chase are shown in Fig. 1. Resolution of the various enkephalin congeners was excellent, and the radioactivity was associated with fractions corresponding to [Met]enkephalin and [Met]enkephalin-Arg^6,Phe^7. To make certain that all the radioactivity in these fractions was really associated with the specific peptides, each was subjected to immunoprecipitation. All of the radioactivity in the tubes corresponding to [Met]enkephalin and [Met]enkephalin-Arg^6,Phe^7 was associated with these peptides (Table 2).

Table 2. Radioactivity (cpm) incorporated into opioid-containing peptides

Time after pulse, hr	22,000-Dalton protein	[Met]Enkephalin	[Met]Enkephalin-Arg^6,Phe^7
0	2660	0	0
6	ND	610	472

Values are for 6 × 10^6 cells. The 22,000-dalton protein value is the lowest estimate from the immunoprecipitation described in Table 1. The [Met]enkephalin and [Met]enkephalin-Arg^6, Phe^7 values are from Fig. 1. ND, not determined.

DISCUSSION

We have shown that in intact chromaffin cells it is possible to label not only the free enkephalins but also at least one of their putative precursors. The other precursors and intermediates (1–3) were not examined in this study. Demonstration of the sequential labeling of this large putative precursor several hours before the appearance of radioactivity in [Met]enkephalin and [Met]enkephalin-Arg,^{6}Phe^7 provides direct evidence that the biosynthesis of the enkephalins occurs via the large precursors. These precursors are then presumably processed via proteases and peptidases. We may suppose that this processing involves several steps. Proteolytic cleavage first yields polypeptides of intermediate size. Peptidic cleavage of these intermediate polypeptides would then yield the smaller peptides. Such a pathway of enkephalin biosynthesis first became apparent from our recent findings of many enkephalin-containing peptides ranging in size from 1000 to >50,000 daltons (1–3). Many of these polypeptides contain more than one enkephalin sequence and therefore represent unusual intermediates. The existence

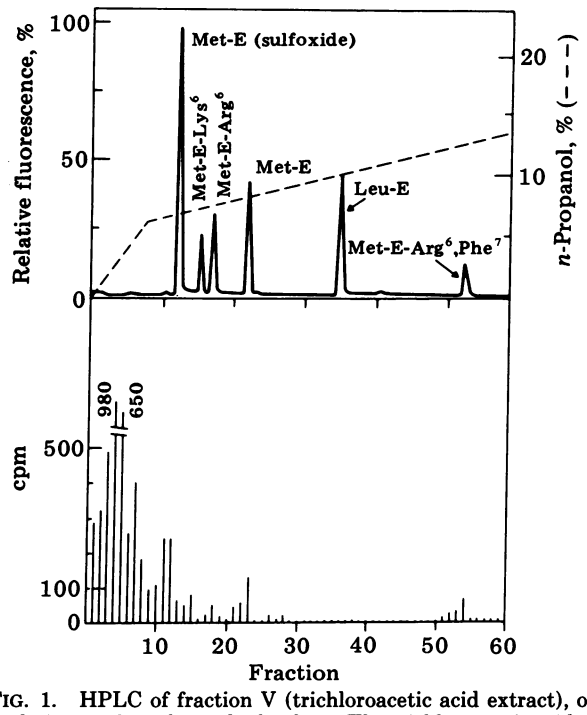

FIG. 1. HPLC of fraction V (trichloroacetic acid extract), obtained after a 2-hr pulse and 6-hr chase. The trichloroacetic acid supernatant was extracted three times with an equal volume of diethyl ether. The pH was adjusted to 4.0 with pyridine. A total of 160,000 cpm was applied to an Ultrasphere octyl column (Altex, Palo Alto, CA). After a 20-min wash with 0.5 M formic acid/0.4 M pyridine, pH 4.0, the column was eluted at 30 ml/hr with a gradient of 0–20% *n*-propanol in the same buffer; 1.5-ml fractions were collected. (*Upper*) Separation of pure peptides as calibrated standards. Elution of the peptides was monitored with a fluorescamine detection system. E, enkephalin. (*Lower*) Radioactivity eluted in each fraction of the experiment is indicated. Results are in cpm/10^6 cells.

of numerous steps in the processing of the large protein precursor may explain the rather long delay in the appearance of radioactive methionine in free enkephalin and the small enkephalin-containing peptides that we have observed here.

The 22,000-dalton compound can now be considered to be an enkephalin precursor. However, the recent report (1) of the existence of a >50,000-dalton enkephalin containing protein indicates that the 22,000-dalton protein is itself an intermediate in the process.

Note Added in Proof. Using cultured chromaffin cells, Wilson *et al.* (8) recently incorporated [^{35}S]methionine into [Met]enkephalin.

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