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CELL-FREE TRANSLATION OF MESSENGER RNA CODING FOR A PRECURSOR OF HUMAN CALCITONIN

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SUMMARY: Polyadenylated RNA, extracted from a human medullary thyroid carcinoma, was translated in cell-free systems prepared from wheat germ and reticulocyte lysates. The major product of the translations was a protein of 15,000 M_R which was immunoprecipitated specifically with an antiserum to synthetic human calcitonin. Addition to the translation reactions of microsomal membranes, prepared from canine pancreas, resulted in the partial disappearance of the 15,000 M_R polypeptide and the concomitant appearance of a smaller peptide (11,000 M_R), also immunoprecipitated specifically by antisera to calcitonin. These results indicate that human calcitonin is synthesized in the form of a precursor of 15,000 M_R and suggest that the precursor contains a leader sequence that is cleaved from the polypeptide by enzymes associated with microsomal membranes.

INTRODUCTION

Calcitonin is a polypeptide hormone of 3500 daltons which regulates calcium metabolism (1,2). In submammalian species, calcitonin is synthesized in a discrete organ, the ultimobranchial gland. Studies of protein synthesis in intact trout (3) and chicken (4) ultimobranchial glands *in vitro* have suggested that calcitonin is synthesized in the form of a larger precursor which is then modified prior to secretion. In higher vertebrates, including man, calcitonin is primarily synthesized by the parafollicular or C-cells which are one of the class of APUD (amine precursor uptake and decarboxylation) cells originating embryologically from the neuroectoderm (5). The diffuse distribution of the C-cells has precluded studies of calcitonin biosynthesis in thyroid glands. To overcome this problem, recent investigations into calcitonin synthesis have made use of tissue obtained from patients with medullary thyroid carcinoma, a tumor of parafollicular cells characterized by marked overproduction of calcitonin. Using this approach, Van der Donk et al. (6) and Lips et al. (7) have reported finding a large precursor of calcitonin of apparent molecular

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weight 65,000 by translation in frog oocytes of mRNA prepared from medullary carcinoma of the thyroid, as well as from a large variety of other tumors.

In the present studies, messenger RNA was extracted from a human medullary thyroid carcinoma and was translated in wheat germ and reticulocyte lysate cell-free systems. The major product of the translations identified by electrophoresis on SDS-polyacrylamide gels was a protein of M_R=15,000 daltons. This protein was immunoprecipitated specifically by antisera to synthetic human calcitonin. Addition to the translation reactions of microsomal membranes prepared from canine pancreas resulted in processing of the 15,000 M_R polypeptide to a smaller peptide (11,000 M_R), also recognized by anti-calcitonin antibodies.

MATERIALS AND METHODS

Preparation of RNA Primary tumor and regional metastases obtained surgically from a patient with histologically proven medullary carcinoma of the thyroid was immediately frozen in liquid nitrogen. Polyadenylated RNA was isolated from the tumor using the methods of Kronenberg et al. (8) and Majzoub et al. (9). Approximately 50 µg (1.0 A_{260} unit) of polyadenylated RNA was obtained from 5 grams of tumor.

Cell-Free Translation Systems Heterologous cell-free translation systems were prepared from extracts of wheat germ (10) and rabbit reticulocyte lysate (11). RNA (0.5-1.0 µg) dissolved in sterile H_2O was translated in reaction mixtures of 25-100 µl containing L-[^{35}S]methionine (500-700 Ci/mmol, 4mCi/ml, New England Nuclear, Boston, MA). After 3 hours of incubation, translation reaction mixtures were adjusted to 0.05M Tris-HCl, pH 7.0, 1% SDS, 1% β-mercaptoethanol, 10% glycerol, and 0.01% bromphenol blue (sample electrophoresis buffer) in preparation for electrophoretic analysis. Stimulation of protein synthesis in the cell-free system was determined by precipitation of labeled proteins in aliquots of the translation reaction mixtures with 10% trichloroacetic acid and determination of radioactivity in the precipitates by liquid scintillation spectroscopy.

Membrane Processing Canine pancreas membranes were prepared by the method of Katz et al. (12). In experiments involving processing of translation products, 1.0 µl (115 A_{280} units/ml) of a suspension of membranes were added directly to the translation mixtures before the addition of RNA.

Immunoprecipitations Immunoprecipitations were performed utilizing a double-antibody procedure. Ten microliters of translation product were added to 100 µl of 10 mM NaH_2PO_4 buffer, pH 7.6, containing 1 mM Na_2EDTA and 1% Triton X-100. Samples were then treated with 1 µl of undiluted antisera (rabbit) prepared against synthetic human calcitonin or non-immune serum and incubated overnight at 4°C. To further test for the specificity of antibody binding, several samples were treated with 10 µg of unlabeled synthetic human calcitonin prior to the addition of antisera. After an additional 18 hour incubation with "second antibody" (sheep antisera prepared against rabbit serum), the immunoprecipitates were centrifuged at 10,000 RPM for 10 minutes

and washed three times with phosphate-buffered saline containing 1% Triton X-100. The immunoprecipitates were dissolved in sample electrophoresis buffer by heating at 37°C for 1 hour and then at 100°C for 2 minutes before electrophoresis on SDS-polyacrylamide gels.

SDS-Polyacrylamide Gel Electrophoresis Translation products and immunoprecipitates were analyzed by electrophoresis on 10 to 20% gradient polyacrylamide gel slabs containing 0.1% SDS (13). Molecular weight markers were subjected to electrophoresis in adjacent wells of the gels. Gel slabs were then stained in 0.2% Coomassie brilliant blue, destained in 25% methanol, 7% acetic acid, and treated with Enhance (New England Nuclear, Boston, MA), an autoradiography enhancer. Autoradiograms were prepared by exposing the vacuum-dried gels to Kodak SB-5 film for periods of one to seven days.

RESULTS

Polyadenylated RNA prepared from a human medullary thyroid carcinoma stimulated protein synthesis in both wheat germ and reticulocyte cell-free systems by 6-fold and 2-fold respectively. In both assays, the major translation product observed by electrophoresis on SDS-polyacrylamide gels was a protein of M_R=15,000 daltons (Fig. 1), considerably larger than authentic calcitonin (MW 3500). The lack of significant incorporation of radioactivity in the absence of mRNA prepared from medullary thyroid carcinoma reflects the dependence of the cell-free translation systems on the addition of exogenous RNA.

The 15,000 M_R translation product was immunoprecipitated with antibody raised to synthetic human calcitonin but not with non-immune serum (Fig. 2). None of the minor translation products were recognized by antisera to calcitonin. Addition of unlabeled synthetic human calcitonin inhibited the immunoprecipitation of radioactive translation product. Antisera to a number of other polypeptide hormones, including ACTH, β-lipotropin, endorphin, and somatostatin did not immunoprecipitate any of the labeled proteins.

When translations were done in the presence of microsomal membranes derived from canine pancreas, the 15,000 M_R protein diminished markedly concomitant with the appearance of a smaller protein of 12,000 M_R (Fig. 3). In addition to the 15,000 M_R protein, the 12,000 M_R product was also immunoprecipitated with antisera raised to human calcitonin but not with

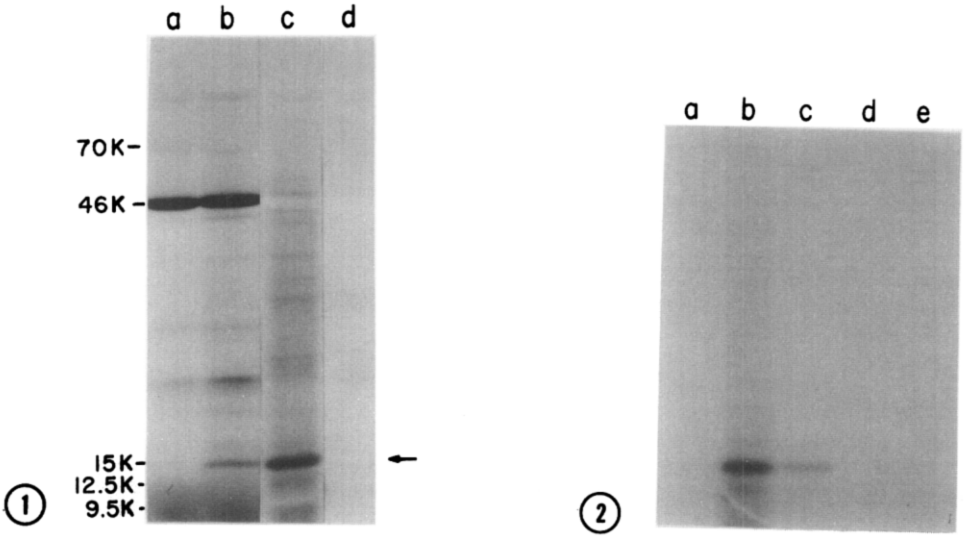

Fig. 1. SDS-polyacrylamide gel electrophoresis of translation products synthesized in wheat germ and reticulocyte cell-free systems. Lane a, reticulocyte lysate, no mRNA added; lane b, reticulocyte lysate, medullary thyroid carcinoma mRNA; lane c, wheat germ, medullary thyroid carcinoma mRNA; lane d, wheat germ, no mRNA. Apparent molecular weights determined by co-electrophoresis with known protein standards; bovine parathyroid secretory protein, 70,000 M_R; actin, 46,000 M_R; pre-proparathyroid hormone 14,000 M_R; bovine heart cytochrome C, 12,400 M_R; parathyroid hormone, 9,500 M_R. Arrow indicates the 15,000 M_R calcitonin-related polypeptide.

Fig. 2. Immunoprecipitations of translation products synthesized in wheat germ cell-free system. Lane a, no mRNA; lane b, human medullary thyroid carcinoma mRNA; lane c, immunoprecipitation with anti-calcitonin antiserum; lane d, immunoprecipitation with anti-calcitonin antiserum after addition of 10 µg of synthetic human calcitonin; lane e, immunoprecipitation with non-immune serum.

non-immune serum. Addition of unlabeled synthetic calcitonin prevented the immunoprecipitation of the 12,000 M_R protein. These observations are consistent with the cleavage by enzymes in the microsomal membranes of a 3000 MW "leader" sequence from the initial translation product (14).

DISCUSSION

These studies show that polyadenylated RNA extracted from human medullary thyroid carcinoma directs the synthesis of a 15,000 M_R protein in wheat germ and reticulocyte cell-free translation systems. Based on the above observations it is likely that this product of 15,000 M_R is a precursor of calcitonin. Further, the identity of the 15,000 M_R translation

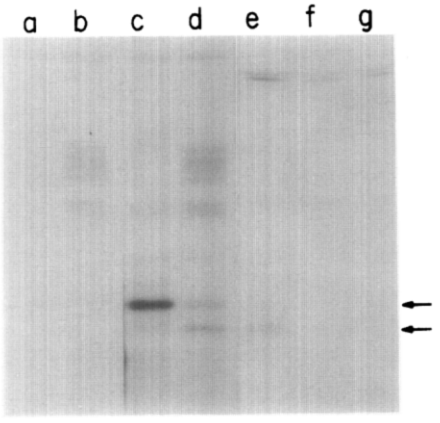

Fig. 3. SDS-polyacrylamide gel electrophoresis of translation products synthesized in wheat germ system after addition of microsomal membranes from canine pancreas. Lane a, no mRNA, no membranes; lane b, membranes, no mRNA; lane c, human medullary thyroid carcinoma mRNA; lane d, mRNA and membranes; lane e, immunoprecipitation with anti-calcitonin antiserum of medullary thyroid carcinoma mRNA translated in the presence of membranes; lane f, immunoprecipitation of mRNA translated with membranes with anti-calcitonin antiserum after addition of unlabeled calcitonin; lane g, immunoprecipitation with non-immune serum of mRNA translated with membranes. Arrows indicate the 15,000 M_R translation product and the 12,000 M_R product which appears after treatment with microsomal membranes.

product as a precursor of human calcitonin is strengthened by the studies reported by Jacobs et al. (15) in which polyadenylated RNA extracted from codfish ultimobranchial glands and also from a rat medullary thyroid tumor directed the synthesis of translation products of similar apparent molecular weight which were also shown to be related to calcitonin by immunoprecipitation.

In the present studies, our failure to detect a translation product of 65,000 daltons related to calcitonin is at variance with the findings of Van der Donk et al. (6) and Lips et al. (7) who reported that the major translation product of mRNA from human medullary thyroid carcinoma observed in frog oocytes was a calcitonin-containing polypeptide of 65,000 M_R. Since the wheat germ and reticulocyte cell-free translation systems cleave the initial translation products in the absence of added microsomal membranes (10,11), the reasons for this discrepancy in results is unclear. Among the possible explanations are: 1) the 65,000 dalton protein is a product only of the frog oocyte translation system, 2) the human

medullary thyroid tumors from which polyadenylated RNA is extracted have intrinsic biosynthetic differences, and 3) the 15,000 M_R precursors may in some manner aggregate to form a product of larger size.

As has been observed in studies of other tumors derived from cells of the APUD series, medullary thyroid carcinoma has been shown to produce a number of polypeptide hormones including ACTH and somatostatin (16,17). Although the particular tumor that we studied was found to contain substantial amounts of immunoreactive ACTH (Leonard Kapcala, personal communication), we were unable to identify an ACTH-related protein in the translation products.

Inasmuch as the putative calcitonin precursor which we have identified is considerably larger than authentic calcitonin (MW=3500), even after its cleavage during mRNA translation in the presence of microsomal membranes, it is possible that the precursor contains sequences of additional peptide hormones as is the case for the 31,000 M_R translation product of the mRNA coding for adrenocorticotropin-lipotropin (18).

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