PARATHYROID mRNA DIRECTS THE SYNTHESIS OF PRE-PROPARATHYROID HORMONE AND PROPARATHYROID HORMONE IN THE KREBS ASCITES CELL-FREE SYSTEM

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SUMMARY

Translation in a cell-free extract of Krebs II ascites cells of a mRNA fraction prepared from bovine parathyroid glands results in the synthesis of two radioactive products that appear identical to pre-proparathyroid hormone (Pre-ProPTH) (M.W. ∿ 14,000), the suspected earliest biosynthetic precursor of parathyroid hormone (PTH) (M.W. 9,500), and to proparathyroid hormone (ProPTH) (M.W. 10,200), the immediate biosynthetic precursor of PTH. The two products of synthesis in the ascites extract co-electrophoresed on both urea—acetate and urea—SDS acrylamide gels with Pre-ProPTH obtained from cell-free translation of parathyroid RNA in extracts of wheat-germ and with ProPTH isolated from parathyroid slices. Both products were precipitated with an antiserum to PTH. Partial analysis of the amino acid sequence of [^{35}S]methionine-labeled Pre-ProPTH synthesized by the ascites extract indicates that a substantial fraction of the product is lacking the two N-terminal methionines present in the Pre-ProPTH synthesized by the wheat-germ system. The results indicate that, (i), unlike the wheat-germ, ascites extracts contain enzymes that remove the initiator methionine from Pre-ProPTH and convert Pre-ProPTH into ProPTH (no ProPTH was observed in the wheat-germ system) and (ii) the cleavage processes appear to occur in association with synthesis, inasmuch as neither removal of NH_2-terminal methionine nor formation of ProPTH was observed upon incubation of Pre-ProPTH isolated from either the wheat-germ system or from the ascites system when put back into the ascites system.

INTRODUCTION

Parathyroid hormone (PTH) is a 84-amino-acid polypeptide that regulates blood calcium levels (1). Its synthesis and secretion, in turn, are controlled principally by the extracellular and/or intracellular concentrations of calcium (1). Studies of PTH biosynthesis in intact parathyroid slices have shown that it is synthesized via a precursor, proparathyroid hormone (ProPTH), of 90 amino acids (2,3). Recently, however, direct translation of a parathyroid mRNA

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SDS: Sodium dodecyl sulfate

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fraction in a cell-free system derived from wheat-germ has yielded a single, major polypeptide of 115 amino acids —— pre-proparathyroid hormone (Pre-ProPTH) (4,5) —— subsequently shown to contain an additional sequence of 25 amino acids at the N-terminus of ProPTH (5,6) and including the initiator NH_2-terminal methionine not removed by the wheat-germ extract (7). Although a polypeptide corresponding to Pre-ProPTH has not yet been identified in intact cell preparations, present evidence indicates that Pre-ProPTH is the initial precursor in the biosynthesis of PTH (4,5).

For further understanding of the mechanisms of PTH biosynthesis, we translated parathyroid mRNA in a heterologous cell-free system derived from Krebs II ascites tumor cells and now report the synthesis of both Pre-ProPTH and ProPTH in the Krebs cell-free system.

METHODS

Preparation of parathyroid RNA: A 8—15 S RNA fraction was prepared from extracts of bovine parathyroid glands (4). The RNA solution (2 mg/ml) was stored in water at -20°C.

Preparation of cell-free extracts: The preparation of cell-free extracts of Krebs II ascites tumor cells (8) and of wheat germ (9) was described previously.

Protein-synthesis assays: Assays in extracts of Krebs ascites tumor cells (S-10) contained in a final volume of 50 µl:10 µl of S-10, 20 mM hepes (pH 7.5), 9 mM 2-mercapto-ethanol, 1.0 mM ATP, 10 mM phosphocreatine, 0.6 mM CTP, 0.1 mM GTP, 0.6 mg/ml creatine phosphokinase, 112 mM KCl, 3.5 mM magnesium acetate, 40 µM unlabeled amino acids, [^{35}S]methionine (Amersham, 350—380 Ci/mM, 8—10 µCi) or [^{3}H]lysine (Amersham, 60 Ci/mM, 10—15 µCi), 4—8 µg parathyroid 8—15 S RNA. Incubations were carried out for 60 min at 37°C. Conditions of the protein-synthesis assay using wheat-germ extract (S-30) were described previously (4). Incubations of parathyroid RNA in the wheat-germ cell-free system were for 3 hr at 22°C. ^{14}C-ProPTH was prepared by electrophoresis of urea—acetic acid extracts prepared from bovine parathyroid gland slices that had been incubated for 30 min in Earle's Balanced Salt Solution containing a mixture of 15 ^{14}C-labeled amino acids (New England Nuclear, 2 µCi/ml) (2,10).

Immunoprecipitation: 15-µl aliquots of the cell-free incubation mixture were incubated for 24 hr with either a guinea-pig antiserum to PTH that reacts with antigenic determinants within both the amino and carboxyl regions of the PTH sequence (11), a control, nonimmune serum, or antiserum to which 2 µg of unlabeled PTH was added. Conditions of the incubation and of the immunoprecipitation of radioactive proteins with a rabbit antiserum to guinea-pig gamma globulin have been described previously (2,10). The immunoprecipitated proteins were dissolved in 8 M urea—0.15 N acetic acid and were analyzed by polyacrylamide gel electrophoresis.

Analysis of radiolabeled proteins: Radiolabeled proteins were extracted from the cell-free reaction mixture and analyzed by electrophoresis on 10% polyacrylamide gels as described previously (2,10). The gels contained either 8 M urea, 0.1 N acetate, pH 4.5 (urea—acetate gels) or 8 M urea, 0.1% SDS, 0.5 M phosphate, pH 7.2 (urea—SDS gels). 1-mm slices of the acrylamide gels were extracted in NCS Solubilizer (Nuclear Chicago), and the radioactivity was counted in Liquifluor (New England Nuclear). Alternatively, [^{35}S]methionine-labeled Pre-ProPTH was recovered from the gel slices by extraction in phosphate—SDS buffer as described previously (4). The recovered ^{35}S-Pre-ProPTH was subjected to a sequential Edman degradation using the Beckman 890 sequenator (12).

[^{35}S]Methionine released during the degradation was identified by thin-layer chromatography (12).

Incubation, in ascites extracts, of Pre-ProPTH isolated from a wheat-germ cell-free system: After a 3-hr incubation of parathyroid RNA and [^{35}S] methionine in a wheat-germ cell-free system, the entire incubation mixture was heated at 50°C for 30 min. This treatment prevented further protein synthesis by wheat-germ polyribosomes. 10 µl of the heated wheat-germ extract plus 100 µM of unlabeled methionine was added to the ascites cell-free system and incubated for 60 min at 37°C. The radioactive products, synthesized originally in the wheat-germ system, were analyzed by electrophoresis on urea—acetate polyacrylamide gels and by sequential Edman degradation. In separate experiments, addition of 10 µl of heat-inactivated wheat-germ mixture alone to the complete ascites cell-free system did not significantly inhibit protein synthesis.

RESULTS

5 µg of parathyroid RNA added to the ascites cell-free system stimulated total protein synthesis by 3- to 4-fold over endogenous synthesis without RNA added (Fig. 1). Analysis by electrophoresis on urea—acetate polyacrylamide gels of the radiolabeled products formed showed the synthesis of two major discrete proteins. One of the proteins co-migrated with ProPTH synthesized by slices of parathyroid glands (Fig. 1). The second protein migrated with Pre-ProPTH synthesized in the wheat-germ cell-free system (Fig 2A). The two major proteins, separately isolated from an urea—acetate gel (Fig. 2A) co-migrated with markers of Pre-ProPTH and of ProPTH when re-electrophoresed on urea—SDS polyacrylamide gels (Figs. 2B,C). Hence, the two proteins co-migrate with markers of Pre-ProPTH and ProPTH on two different gel systems:

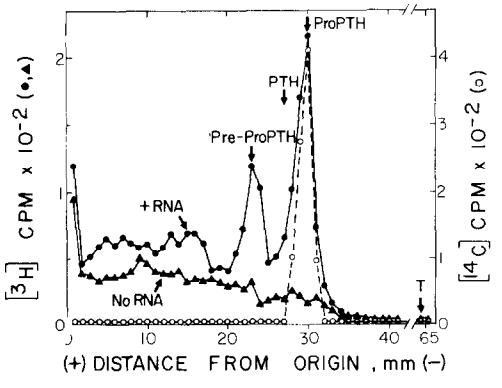


Figure 1. Electrophoretic profiles (urea—acetate polyacrylamide gels) of [^{3}H]lysine-labeled products synthesized in response to 8—15 S fraction of parathyroid RNA in a Krebs II ascites cell-free system. [^{3}H]Lysine-labeled cell-free products formed with (●) and without (▲) added parathyroid RNA (co-plotted). [^{14}C]Labeled ProPTH isolated from parathyroid slices was added to the triated peptides as a marker (○). Arrows indicate electrophoretic positions of Pre-ProPTH, ProPTH, PTH and T, the position of methyl-green tracker dye. Migration from left to right.

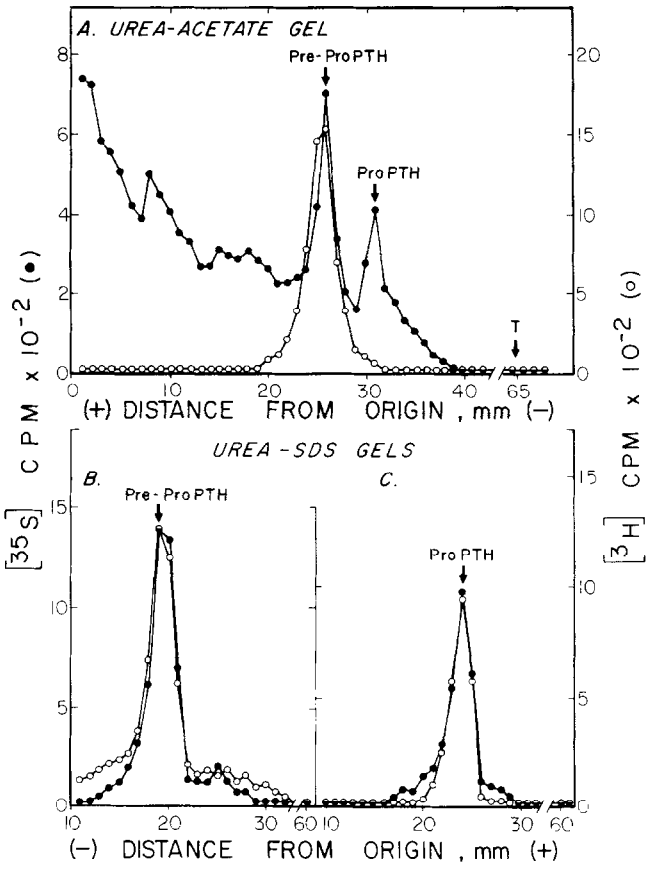
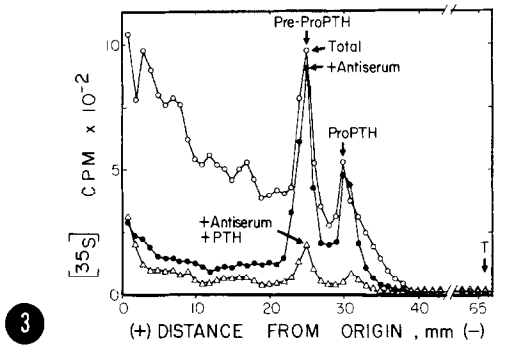


Figure 2. Electrophoretic profiles of [^{35}S]methionine-labeled products directed by parathyroid RNA in an ascites cell-free system (●). (A), urea—acetate polyacrylamide gel of cell-free products with [^{3}H]lysine-labeled Pre-ProPTH synthesized in wheat-germ cell-free system added to [^{35}S]methionine-labeled products as a marker (○). (B) and (C), urea—SDS polyacrylamide gel electrophoresis of [^{35}S]methionine labeled Pre-ProPTH (B), and [^{35}S]methionine-labeled ProPTH (C) isolated from urea—acetate gel electrophoresis identical to that shown in (A). [^{3}H]Lysine-labeled products (Pre-ProPTH) from wheat-germ cell-free system was co-added in (B) and [^{3}H]leucine-labeled ProPTH was co-electrophoresed in (C).

urea—acetate gels, which separate proteins primarily on the basis of their inherent net charge (13), and urea—SDS gels, on which separation is a function of the molecular weights of the proteins (14).

Electrophoresis of immunoprecipitates prepared from the ascites incubation mixture treated with either antiserum to PTH, control nonimmune serum, or antiserum to PTH absorbed with excess unlabeled PTH are shown in Fig. 3. The results demonstrate specific binding, by the antiserum, of both proteins (Fig. 3)

Further evidence that a major fraction of the synthesized larger pro-



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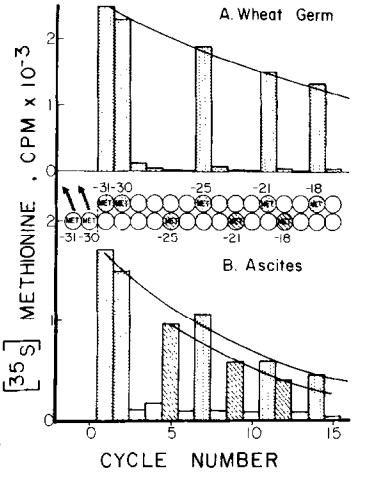

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Figure 3. Urea-acetate polyacrylamide gel profiles of immunoprecipitates prepared from [^{35}S]methionine-labeled cell-free products synthesized in Krebs ascites cell-free system in response to parathyroid RNA. (O), aliquot of total cell-free incubation mixture. Products bound by antiserum to PTH with (Δ) and without (●) addition of excess of unlabeled PTH. Profile of radioactive products treated with nonimmune control serum (not shown) was similar to that of antiserum with excess PTH added.

Figure 4. Sequential Edman degradation of [^{35}S]methionine-labeled Pre-ProPTH isolated from parathyroid RNA translation products in cell-free systems derived from wheat germ (A) and Krebs II ascites tumor cells (B). Bars denote ^{35}S radioactivity released at each cycle of the degradation. Repetitive yields of amino acids released from apomyoglobin carrier at each step were determined by gas chromatography and the expected yield of radioactivity at each cycle relative to the initial peak is indicated by the curved lines. Note [^{35}S]methionine (B) released at cycles 5,9,12 (cross-hatched bars) consistent with lower sequence alignment (terminal methionines removed via cleavage) in addition to pattern of radioactivity seen at cycles 1,2,7,11,14 with alternate upper sequence alignment representing intact Pre-ProPTH also present in ascites system and solely present (A) in wheat-germ system (stippled bars).

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tein is identical to Pre-ProPTH synthesized in the wheat-germ cell-free system was obtained by sequential Edman degradation of the protein labeled with [^{35}S]methionine (Fig. 4). [^{35}S]Methionine was released at cycles 1,2,5,7,9,11, 12, and 14. Analysis of the kinetics of the sequential degradation, determined by monitoring the repetitive yields of amino acids released at each cycle from apomyoglobin added as a carrier to the radioactive protein (12), indicates that two separate proteins were simultaneously undergoing sequential degradation. The major protein yielded methionines at cycles 1,2,7,11, and 14 (stippled bars), identical to the sequence of methionines released from Pre-ProPTH synthesized by wheat-germ extracts. The other protein yielded methionines at cycles 5, 9, and 12 (cross-hatched bars), suggesting that this

protein differs from Pre-ProPTH in that it is lacking the Met—Met sequences at the NH_2-terminus of the polypeptide (Fig. 4).

Analysis of the amino-acid sequence of [^{35}S]methionine-labeled products from the wheat-germ cell-free system both before and after their incubation in the ascites cell-free system showed that the pattern of [^{35}S]methionine released was similar to that shown in Figure 4A. No step-up in radioactivity was observed at cycles 5,9, and 12.

DISCUSSION

RNA from bovine parathyroid glands directs the synthesis of both Pre-ProPTH and ProPTH in a cell-free system containing extracts of Krebs II ascites tumor cells. The major product synthesized in the ascites cell-free system is ProPTH. The use of lysine (Fig. 1) rather than methionine (Fig. 2A) as the radioactive amino acid precursor provides a more accurate assessment of the amount of ProPTH (75%) synthesized relative to the amount of Pre-ProPTH (25%), inasmuch as ProPTH (90 amino acids) and Pre-ProPTH contain 12 and 14 lysines compared with 2 and 7 methionines, respectively (7,15). The products from the translation of parathyroid mRNA in the ascites cell-free system differ from those found upon similar translations in a cell-free system derived from wheat-germ extract where only Pre-ProPTH is synthesized and no ProPTH is found (4,6,7). Insofar as Pre-ProPTH may be a biosynthetic precursor of ProPTH (4,5), it appears that the ascites system, in contrast to the wheat-germ system, contains the specific proteolytic activity required for the conversion of Pre-ProPTH to ProPTH.

An alternative explanation for our results is that Pre-ProPTH and ProPTH are the products of separate genes and that the mRNA for ProPTH is translated by the ascites, but not by the wheat-germ, cell-free systems. This explanation seems unlikely in view of the very high efficiency of the wheat-germ system in translating mRNA's from a variety of sources (9).

Other workers also have found differences in the cleavage activity between ascites and wheat-germ cell-free systems. Heavier proteins, thought to represent biosynthetic precursors, have been identified upon translations in the wheat-germ system of RNA for human placental lactogen (16), human growth hormone (17), and immunoglobulin light chains (16), whereas the smaller authentic proteins were the major species found after translations of these same RNA's in the ascites cell-free system (16,18,19).

Our studies additionally indicate that the ascites extract but not the wheat-germ extract contains aminopeptidase activity that specifically removes N-terminal methionines, including the initiator methionine, from the PTH polypeptides undergoing synthesis. The Met—Met sequence was found to be absent from the N-terminus of a substantial fraction of the Pre-ProPTH synthesized

in the ascites system. Previously, we showed that Pre-ProPTH synthesized in the wheat-germ system begins with the sequence Met—Met and that the NH_2-terminal methionine is incorporated uniquely into the polypeptide by initiator methionyl tRNA (7).

Our finding that no cleavages occurred in Pre-ProPTH prepared from wheat-germ extract upon its incubation in active extracts of ascites cells further suggests that the proteolytic enzymes responsible for these specific cleavages of Pre-ProPTH may be discretely localized in the biosynthetic pathway, for example, within particulate moieties (ribosomes, endoplasmic reticulum) involved in the polymerization of amino acids and the subsequent transport of the completed polypeptide chains into the cisterna of the endoplasmic reticulum (20). Intratranslational and rapid post-translational cleavages of proteins are already known to occur. The N-terminal initiator methionines of proteins are usually removed from nascent polypeptide chains before completion of their synthesis (21). The translation products of polio-virus RNA are thought to undergo proteolytic cleavages during the growth of the polypeptide chain (22). The failure thus far to identify Pre-ProPTH in studies of PTH biosynthesis in intact parathyroid tissue (4) suggests that Pre-ProPTH in vivo also may rapidly cleave to ProPTH either during or immediately after completion of the synthesis of the polypeptide chain in the rough endoplasmic reticulum.

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