PARTIAL AMINO ACID SEQUENCE OF HUMAN PLACENTAL LACTOGEN PRECURSOR AND ITS MATURE HORMONE FORM PRODUCED BY MEMBRANE-ASSOCIATED ENZYME ACTIVITY

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SUMMARY

The precursor form of human placental lactogen, synthesized by a wheat germ extract cell-free system, has been partially sequenced and found to contain a high percentage of leucine residues within its first 20 amino acids. The partial NH_2-terminal structure appears to be:

MET	PRO	X	X	X	X	X	LEU	LEU	LEU	X	X	X	LEU	LEU	X	LEU	X	PRO
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

The precursor form of hPL, produced by an ascites extract cell-free system, was cleaved by a membrane-associated enzyme into a form which exhibits the methionine and valine residues in NH_2-terminal positions identical to those of native human placental lactogen.

INTRODUCTION

The precursor forms of several secretory proteins, synthesized *in vitro*, have been shown to contain approximately 25 amino acids at the NH_2-terminus comprising the so-called precursor piece (1-4). While this NH_2-terminal sequence is apparently different in the various precursors, one common characteristic is a high content of hydrophobic amino acids that might facilitate binding to the endoplasmic reticulum. While the function of these precursor forms has not been fully assessed, it has been suggested that the extra protein portion may play a role in mediating the formation of "membrane-bound" ribosomal complexes (5, 6).

Recently, it has been shown that a precursor of human placental lactogen (prehPL), synthesized in ascites tumor cell-free extracts, can be converted into a molecule resembling native human placental lactogen (hPL) by an ascites

Abbreviations: hPL, human placental lactogen; PITC, phenylisothiocyanate; TLC, thin-layer chromatography; PTH, parathyroid hormone.

tumor membrane fraction that includes membranes derived from the endoplasmic reticulum (7). The identification of this cellular fraction, which contains the cleavage enzyme, has permitted us to study the amino acid sequence of prehPL prior to and following cleavage to determine the specificity of the enzyme for this substrate. In this paper we present amino acid sequence evidence indicating (1) that the precursor piece of prehPL is located at the NH_2-terminus of the molecule and contains a particularly high percentage of leucine residues; (2) that the membrane-associated cleavage activity is indeed specific for the production of hPL from prehPL, i.e., it converts prehPL into a molecule with the NH_2-terminal sequence of the native hormone.

MATERIALS AND METHODS

Cell-Free Synthesis

The preparation of wheat germ extracts, cell-sap (S-100), ribosomes, and membrane fractions derived from Krebs II ascites tumor cells has been described previously (7). Term placental mRNA was isolated according to Boime et al.(8).

The products synthesized in vitro were eluted from 20% preparative polyacrylamide slab gels (7). All experiments incorporating a tritiated amino acid also included ^{35}S-methionine so that the appropriate bands could be identified by autoradiography. The labelled proteins were synthesized in 1.2 ml reaction mixtures containing 1.4 µM ^{35}S-methionine, and 10 µM of the indicated (^{3}H) amino acid; the remaining components for reaction mixtures containing either wheat germ or ascites extracts have been described (7).

Determination of Amino Acid Sequence

Purified cell-free product was freeze dried. Five mg of sperm whale apomyoglobin (Beckman No. 339182) was dissolved in 500 µl of trifluoroacetic acid (Pierce Chem. Co.) and transferred to the tube containing the lyophilized product, which readily dissolved. After removal of an aliquot for determination of total radioactivity, the product was transferred to the cup of a Beckman updated model 890B sequencer and dried at low speed. The sample was subjected to one degradation cycle without PITC or quadrol. This aided in removal of residual acrylamide from the reaction cup. Beckman program number 060275 was used as well as Beckman sequencer reagents.

The thiazolinones, dissolved in chlorobutane, were dried down under nitrogen, resuspended in 500 µl of ethyl acetate and 50 µl removed for counting in a xylene based cocktail in a Packard Model 3375 liquid scintillation counter. The remainder of each thiazolinone was again dried under nitrogen and converted to its phenylthiohydantoin in 1N HCl at 80°C for 10 min. The PTH-amino acids were extracted with ethyl acetate (2 x 1 ml), dried down and resuspended in 50 µl of ethylene dichloride, 10 µl of which was also counted. In addition a portion of the aqueous phase was counted in Instabray (Yorktown). All preparations contained a double label of ^{35}S-methionine and another ^{3}H-amino acid. All determinations were corrected for ^{35}S overlap into the tritium channel. In order to monitor the sequencer for proper operation the first 8 steps of the myoglobin carrier were confirmed on the Edman "H" TLC system (9). Repetitive yield was determined by hydrolysis of the myoglobin carrier at steps 2, 9, and 11 (leucines) with HI (10) followed by amino acid analysis on a Beckman Model 121. The repetitive yield of the apomyoglobin carrier was 91-94%.

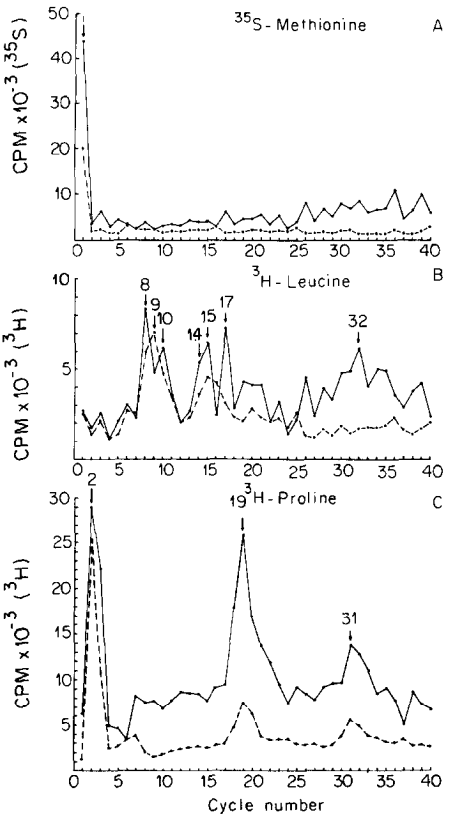

Fig. 1. Radioactivity recovered as thiazolinones following automated Edman degradation of prehPL synthesized in a wheat germ cell-free system labelled with: (A) ^{35}S-methionine (B) ^{3}H-leucine and (C) ^{3}H-proline. Two runs are shown for each product (solid line and dashed line). Positions of residues are indicated by number and arrow. Similar analyses of organic phases and thin layer chromatography of the PTH-amino acids confirmed the presence of methionines, leucines, and prolines at the positions indicated above.

The identification of the radiolabelled amino acids was confirmed by thin layer chromatography on silica gel in the Edman "D" system with standard PTH-amino acid carriers. The PTH-amino acid carrier spots were scraped off the silica gel plates and counted in the liquid scintillator.

RESULTS AND DISCUSSION

All preparations of precursor from the wheat germ system or the ascites system displayed ^{35}S-methionine radioactivity only at cycle 1. This is illustrated in Fig. 1A. No other labelled methionine residues were detected in the first 30 positions leading to the conclusion that methionine occupies only the

NH_2-terminus, i.e., in contrast to "prepro" PTH, methionine is not present elsewhere in the precursor segment of hPL (1). The yield of radioactivity at position 1, relative to the total amount loaded into the sequencer cup, was 3-4%, which is a 21-28% yield assuming the presence of 7 methionines in the entire molecule.

Figures 1B and 1C depict the distribution of ^{3}H-leucine and ^{3}H proline respectively in the prehPL synthesized by the wheat germ system. Peaks of ^{3}H-leucine were observed at positions 8, 9, 10, 14, 15, 17 and less definitively at position 32. This pattern of leucine residues is similar to that observed in the immunoglobulin precursors (3). Automated Edman degradation of ^{3}H-proline labelled prehPL indicated the presence of proline at positions 2, 19 and 31. Similar sequencing of prehPL labelled with ^{3}H-valine did not reveal the presence of any valines within the first 25 residues but some radioactivity was noted in the wheat germ preparation at residues 27-31. Native hPL contains valine at positions 1 and 4, proline at 5, and leucine at 6. If the precursor portion of prehPL consists of 26 amino acid residues., valine should appear at positions 27 and 30, proline at 31 and leucine at 32 in the precursor form. The presence of proline at position 31 of prehPL is clear (Fig. 1C), but the data for valine and leucine positions in this region are not conclusive enough to establish the size of the precursor piece. The partial sequence of the precursor region determined thus far appears to be: $\underset{1}{\text{MET}}\text{-}\underset{2}{\text{PRO}}\text{-}\underset{3}{\text{X}}\text{-}\underset{4}{\text{X}}\text{-}\underset{5}{\text{X}}\text{-}\underset{6}{\text{X}}\text{-}\underset{7}{\text{X}}\text{-}\underset{8}{\text{LEU}}\text{-}\underset{9}{\text{LEU}}\text{-}\underset{10}{\text{LEU}}\text{-}\underset{11}{\text{X}}\text{-}\underset{12}{\text{X}}\text{-}\underset{13}{\text{X}}\text{-}$

$\underset{14}{\text{LEU}}\text{-}\underset{15}{\text{LEU}}\text{-}\underset{16}{\text{X}}\text{-}\underset{17}{\text{LEU}}\text{-}\underset{18}{\text{X}}\text{-}\underset{19}{\text{PRO}}.$

HPL m-RNA directed the synthesis of prehPL in an ascites cell-free system containing ribosomes and S-100. When an ascites membrane fraction was added to this cell-free system at the start of the reaction, the product was a protein which migrated with hPL and also contained authentic hPL peptides as shown by fingerprints (7). In the experiments reported here ascites synthetic reaction mixtures containing ^{35}S-methionine, mRNA, ribosomes, and S-100, incubated in the presence and absence of a membrane fraction, were applied to preparative polyacrylamide gels. The prehPL (A) and hPL (B) bands were eluted and subjected to

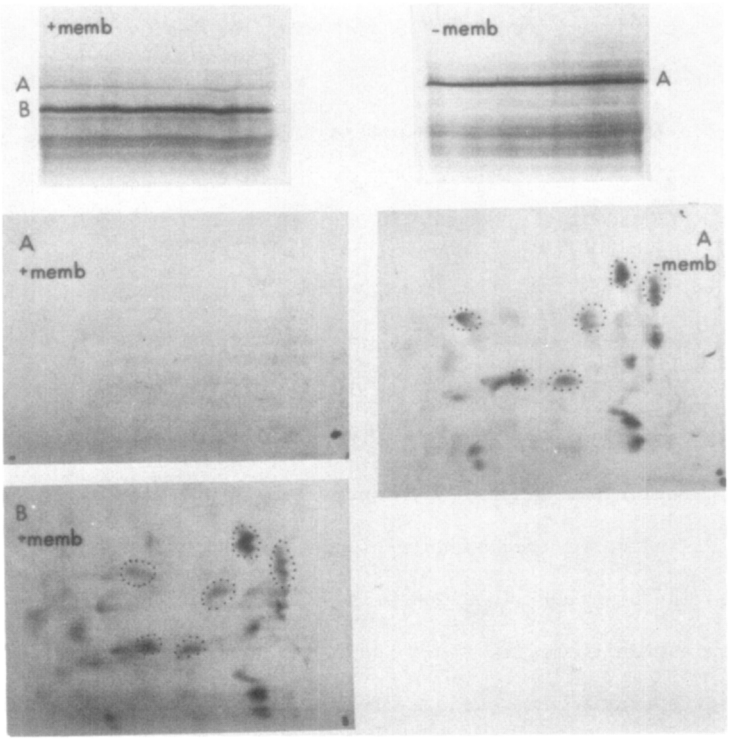

Fig. 2. Two dimensional tryptic fingerprints of prehPL and its cleaved product (from the cell-free ascites system) eluted from preparative polyacrylamide gels. Approximately 1.3 x 10^6 cpm of labelled proteins synthesized in the presence and absence of membranes were applied to a preparative acrylamide gel that was divided into two lanes. A and B denote prehPL and hPL respectively, and these designated bands were eluted and fingerprinted. The peptides enclosed by dotted rings on the autoradiograms represent the 6 methionine-containing tryptic peptides in purified hPL. Products prepared were subjected to NH_2-terminal sequence analysis (see Fig. 3) by the same method.

tryptic fingerprint analysis as shown in Fig. 2. Both the prehPL synthesized in the absence of membranes and the hPL synthesized in the presence of membranes which were subjected to sequence analyses contained methionine-labelled tryptic peptides that correspond to authentic hPL tryptic peptides as previously reported (7, 8). In order to determine how much of the NH_2-terminal region was removed by the membrane-cleavage activity, the NH_2-terminal amino acid positions of radioactive methionine and valine residues were determined in the uncleaved and cleaved products. Figure 3(A-D), contains the results of this study. The prehPL contains

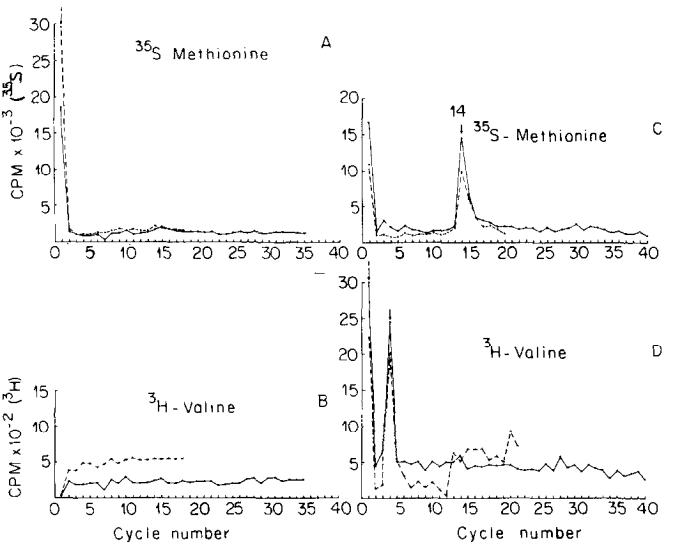

Fig. 3. Patterns of radioactivity recovered at each step (as thiazolinones) from automated Edman degradation of ^{35}S-methionine and ^{3}H-valine labelled products of the ascites cell-free system. A and B are prehPL (see Fig. 2, product A) while C and D are the membrane cleaved hPL products (see Fig. 2, product B). The ^{35}S-methionine counts at position one in panel C are presumably due either to contaminating proteins which have NH_2-terminal methionines as the initiator amino acid or uncleaved precursor. Two runs are shown (dotted and dashed lines).

^{35}S-methionine at position 1 and has no ^{3}H-valine residues within the first 25 steps. The hPL membrane-cleaved product has ^{35}S-methionine mainly at step 14 and has ^{3}H-valine residues at positions 1 and 4, all three of which are identical to the sequence of native hPL (11). The presence of some ^{35}S-methionine at step 1 in the hPL product is likely to be due to contamination with uncleaved proteins starting with methionine.

The data presented in this paper indicate that prehPL forms produced by cell-free systems derived from either wheat germ or ascites extracts contain methionine at the NH_2-terminus and lack the valines that are known to be near the NH_2-terminus of native hPL. In addition, the precursor preparations produced by the wheat germ system have been shown to be particularly rich in leucine. It has also been shown that membrane bound enzyme activity cleaves this prehPL form into a product that is apparently the same as native hPL. No intermediate

precursor such as the "pro-parathyroid hormone" (1) was detected by the methods used in these studies.

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