Synthesis of Preprolactin and Conversion to Prolactin in Intact Cells and a Cell-free System*  

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SUMMARY

Monolayer cultures of pituitary cells were pulse-labeled with [3H]leucine for several minutes and the incorporated radioactivity was analyzed by immunoprecipitation and electrophoresis on sodium dodecyl sulfate containing polyacrylamide gels. Following a 3-min labeling period, a peak of radioactivity with a mobility similar to that of preprolactin was observed, as well as radioactivity co-migrating with prolactin. Competition with unlabeled prolactin demonstrated the specificity of the immunoprecipitation reaction. After 5 min of pulse-labeling followed by 5-min chase in medium with unlabeled leucine, only a product with the mobility of prolactin remained. Addition of a membrane fraction from dog pancreas to a wheat germ cell-free translation system containing pituitary mRNA resulted in the conversion of preprolactin to prolactin. Partial sequence analysis demonstrated that the processed product contained the correct NH$_2$ terminus of prolactin. Thus, both intact pituitary cells and a cell-free heterologous system are able to synthesize preprolactin and cleave it to prolactin offering strong evidence that preprolactin is the biosynthetic precursor to prolactin.

The in vitro translation products of a number of secretory proteins have been found to be larger than the normally secreted form of the protein (for references see Ref. 1). The large cell-free translation products have been termed preproteins and it has been assumed that they represent precursors of the secretory proteins. However, it has been difficult to obtain evidence that the preproteins are actually synthesized in intact cells, presumably because they are very rapidly cleaved to a relatively stable intermediate storage size (proprotein) or to the normal secretory size of the protein. Indeed, experiments with polysomes have suggested that preproteins are cleaved before the nascent peptide is completed (2-5). This would suggest that it would be impossible to detect complete preproteins in intact cells. However, pulse-chase studies have demonstrated the synthesis of preproparathyroid hormone (6) and preproinsulin (7) in intact cells. Detection of these preproteins in intact cells might be related to their small size of approximately 100 amino acids. Perhaps a protease removes the precursor segment when the nascent peptide is 50 to 100 amino acids long. Thus, precursors of about this size might be synthesized complete and then cleaved. However, in the present report we demonstrate that preprolactin which is twice as large as preproparathyroid hormone or preproinsulin is synthesized in intact pituitary cells. Also, a cell-free protein-synthesizing system containing a heterologous membrane fraction accurately cleaved preprolactin to prolactin, offering further evidence that preprolactin is the biosynthetic precursor to prolactin.

EXPERIMENTAL PROCEDURES

Cell Culture—Pituitaries from female, Sprague-Dawley rats were dispersed by treatment with 0.3% collagenase and 0.25% trypsin as described by Vale et al. (8). Dispersed cells were added to 35-mm tissue culture dishes in 2 ml of Minimal Essential Medium/d-glucose (Gibco, Grand Island, NY) containing 10% dialyzed fetal calf serum and maintained at 37°C in a 5% CO$_2$ atmosphere. After 2 days, the medium was renewed and the cells were incubated for 15 min at 37°C in Minimal Essential Medium (without leucine). This medium was replaced with 0.5 ml of Minimal Essential Medium containing 3 mg/liter of leucine + 20 μCi/ml of [3H]leucine (50 Ci/mmol). Following labeling, the medium was removed and the cells were rinsed with ice cold phosphate-buffered saline containing 0.01 M leucine and then centrifuged in 0.2 ml of NaCl/P$_4$ containing 1% Triton X-100, 1% deoxycholate and 0.01 M leucine. Cells were homogenized in a Tedlar glass homogenizer and centrifuged at 10,000 g for 10 min prior to immunoprecipitation.

Cell-free Protein Synthesis—Pituitary RNA was prepared and translated in wheat germ extracts as described previously (9, 10). Dog pancreatic membranes were prepared and stored as described by Katz et al. (11). Following cell-free protein synthesis, reactions were diluted with an equal volume of NaCl/P$_4$, 2% Triton X-100, 2% deoxycholate, 0.02 M leucine and then centrifuged at 10,000 g × 10 min. Aliquots of the 10,000 g supernatant were analyzed by immunoprecipitation.

Immunoprecipitation and Analysis on Gels—For carrier immunoprecipitation, 5 μg of [3H]prolactin were added to each sample and then an amount of specific rabbit anti-prolactin in excess of that required to precipitate the added carrier prolactin (8). After incubation overnight at 4°C, the immunoprecipitate was washed and pelleted as described previously (12). Previous studies have shown that the antisera used in this study are monospecific for prolactin (9, 12). For analysis on gels, the pellet was dissolved in 100 μl of 1% sodium dodecyl sulfate, 4 M urea, 0.05 M Tris- HCl, pH 7.4, 1% β-mercaptoethanol and heated to 90°C for 5 min. The sample was then electrophoresed on a 0.6 × 9 cm sodium dodecyl sulfate containing 12% polyacrylamide, 1.2% N,N-diallyltartardiamide gel using a discontinuous Tris/glycine buffer system (13). The gels were sliced and incubated overnight in 2% periodic acid, and the radioactivity was determined after addition of scintillation fluid.

Alternatively, samples were combined with 0.02 ml of rabbit antiserum to rat prolactin and incubated overnight at 4°C. Then, 0.02 ml of a 10% suspension of Staphylococcus aureus, Cowan 1 strain, ATCC No. 12595, prepared as described by Kessler (14), was added to the samples and incubated for 15 min at 4°C. Antigen antibody complexes adsorbed to the Staphylococcus aureus were collected by centrifugation at 5,000 × g for 10 min. The pellet was washed four times by suspension in 0.75 ml of NaCl/P$_4$, 1% Triton X-100, 1% deoxycholate, 0.01 M leucine followed by centrifugation at 2,000 × g for 10 min and transferred to a new tube for a final wash. The immunoprecipitated radioactivity was released from the pellet by incubation in 1% sodium dodecyl sulfate, 4 M urea, 0.05 M Tris, pH 7.4, 1% β-mercaptoethanol for 5 min at 90°C. After centrifugation at 10,000 × g for 10 min, the supernatant was analyzed on sodium dodecyl sulfate-containing polyacrylamide gels as described above.

Sequence Analysis—Cell-free products synthesized in the presence of Iden...
of dog pancreas membranes were labeled with $[^{35}S]$cystine (40 Ci/mmol) and $[^{3}H]$glycine (23 Ci/mmol). The cell-free product was immunoprecipitated in the presence of carrier prolactin and subjected to sequence analysis as described previously (10).

RESULTS

Synthesis of Preprolactin in Intact Cells—Monolayer cultures of dispersed pituitaries were used in an effort to detect the synthesis of preprolactin in intact cells. Analysis by polyacrylamide gel electrophoresis of products labeled by a 3-min pulse with $[^{3}H]$leucine demonstrated the synthesis of several peaks of immunoreactive radioactivity (Fig. 1A). The major peak of radioactivity co-migrates with a prolactin standard. A peak of more slowly migrating radioactivity has an electrophoretic mobility similar to that of preprolactin synthesized in the cell-free wheat germ system (for comparison, see Fig. 3). The radioactivity which migrates more rapidly than prolactin probably represents incomplete peptide chains. The effects of competition with prolactin for binding to the antibody were next examined in order to be certain that all of the immunoreactive material synthesized during the short pulse actually contained the antigenic determinants of prolactin. Addition of unlabeled prolactin to the immunoprecipitation reaction almost completely abolished the binding of radioactivity to the antibody (Fig. 1B). These results strongly suggest the synthesis of preprolactin in intact pituitary cells.

In order to examine the fate of preprolactin synthesized in intact cells, a brief labeling period (5 min) was followed by a chase (5 min) with unlabeled amino acids. Without the chase, the radioactivity is again found in preprolactin, prolactin, and some peptides (Fig. 2A). Following the chase period, radioactivity was only found in prolactin (Fig. 2B). Thus the 5-min chase period appears to be sufficient to complete elongation of nascent chains and remove the precursor segments of all peptides labeled during the initial period. These findings support the view that preprolactin is a precursor of prolactin.

Conversion of Preprolactin to Prolactin in a Cell-free System—As described previously (9, 15, 16), translation of pituitary RNA in a cell-free system from wheat germ results in the synthesis of preprolactin (Fig. 3A). Addition of a membrane preparation from dog pancreas to the wheat germ reaction mixture resulted in the synthesis of a substantial amount of cell-free product which co-migrates with prolactin (Fig. 3B). The membrane preparation apparently cleaved preprolactin to a size similar to that of prolactin. Addition of membranes to the wheat germ reaction mixture after completion of translation did not alter the mobility of preprolactin (data not shown). The fidelity of this apparent cleavage was examined by sequencing cell-free products synthesized in the presence of dog pancreas membranes and labeled with $[^{3}H]$glycine and $[^{35}S]$cystine. The NH$_2$ terminus of the processed material contains cysteine at positions 4 and 9 and glycine at positions 6 and 7 (Fig. 4). This is identical to the sequence of these amino acids at the NH$_2$ terminus of prolactin. The data also suggest the presence of a second sequence with a glycine at position 11. This aligns with the sequence of preprolactin and is consistent with the observation that not all of the

Fig. 1. Polyacrylamide gel electrophoresis of prolactin immunoreactive material synthesized when pituitary cells were incubated with $[^{3}H]$leucine for 3 min. A, the 10,000 x g supernatant of the homogenized cells was incubated with anti-prolactin overnight and the immunoprecipitate was isolated by adsorption to Staphylococcus aureus. The immunoprecipitate was released from the S. aureus by incubation for 5 min at 90°C in 4 M urea, 1% sodium dodecyl sulfate, 0.01 M Tris, pH 7.4. Prior to electrophoresis on a 10% polyacrylamide gel, the sample was combined with a $[^{14}C]$prolactin standard. B, a cell extract was incubated with anti-prolactin plus 100 pg of unlabeled rat prolactin and then processed as above. - - - [H]labeled preprolactin (PREPRL), and prolactin (PRL); - - - [35]S]cystine standard.

Fig. 2. Processing of preprolactin (PREPRL) in intact cells. A, pituitary cells were incubated with $[^{3}H]$leucine for 5 min. B, pituitary cells were incubated with $[^{3}H]$leucine for 5 min followed by 5-min chase incubation in medium with unlabeled leucine. The 10,000 x g supernatant of the homogenized cells was immunoprecipitated by the addition of carrier $[^{13}C]$prolactin and anti-prolactin. The immunoprecipitate was dissolved in sodium dodecyl sulfate-containing buffer and analyzed on a 12% polyacrylamide gel.

$^{2}$D. J. McKean and R. A. Maurer, manuscript in preparation.
Preprolactin Synthesis and Conversion

A No membranes

B Plus dog pancreas membranes

**FIG. 3.** Processing of preprolactin (PREPRL) in a cell-free wheat germ protein-synthesizing system containing dog pancreas membranes. A, pituitary RNA was translated in a 50-μl wheat germ reaction containing [3H]leucine. B, pituitary RNA was translated in a 50-μl wheat germ assay containing [3H]leucine and 5 μl of a dog pancreas membrane preparation. The cell-free reactions were immunoprecipitated and analyzed on polyacrylamide gels as in Fig. 2.

preprolactin is cleaved to prolactin by the dog pancreas membranes.

**DISCUSSION**

The present findings demonstrate that preprolactin is synthesized in pituitary cells and suggests that preprolactin is the biosynthetic precursor of prolactin. Furthermore, the demonstration of a cell-free system which synthesizes preprolactin and accurately cleaves it to prolactin is consistent with this view.

The function of the precursor segment of the preproteins is not known. The "signal hypothesis" suggests that the precursor segment is involved in recognition of endoplasmic reticulum and transport of secretory proteins (2). The recent finding that the primary translation product of ovalbumin mRNA does not contain a precursor segment demonstrates that not all secretory proteins contain such a peptide (17). At this time, it is not clear if ovalbumin is merely an exception which is secreted by an unusual mechanism or if the precursor segment is not involved in the secretory process.

Preprolactin synthesis was detected by pulse-labeling pituitary cells in monolayer cultures. Use of monolayer cultures allows the rapid disruption of cell membranes with detergents. As the enzyme which cleaves the precursor segment appears to be localized in a membrane fraction, rapid disruption of membranes may facilitate the ability to detect the synthesis of preproteins. Pulse-labeling of pituitary fragments rather than cell cultures failed to demonstrate the synthesis of preprolactin. It is possible that the time required to wash and homogenize tissue fragments allows the cleavage of preprolactin.

Although our studies demonstrate the synthesis of apparently complete preprolactin in intact cells, it has not been possible to determine if cleavage of preprolactin always occurs after completion of synthesis. Cleavage of the precursor segment could possibly occur at random times during the synthesis of a polypeptide. Thus, some polypeptides might be synthesized as the complete preprotein and then cleaved, while the precursor segment might be removed before the completion of other polypeptides.

A heterologous cell-free system containing dog pancreas membranes and wheat germ extract is able to accurately cleave preprolactin to prolactin. Presently the enzyme recognition site which allows this accurate cleavage is not apparent. The amino acid sequence of preprolactin immediately preceding the prolactin NH₂ terminus differs from that of other precursors. Analysis of the enzyme mechanisms which lead to the accurate removal of the precursor segment of secretory proteins will likely require development of a direct enzyme assay.

Addition of membranes to cell-free translation systems results in cleavage of preprolactin or other preproteins only when membranes are present during translation, not when they are present after translation (2, 3, 18, 19). This has led to the suggestion that only nascent chains can be cleaved (19). However, the finding that intact cells synthesize the complete preprolactin molecule which is subsequently cleaved to prolactin raises the possibility that cleavage can occur following

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3 R. A. Maurer, unpublished observations.
the release of preproteins from the ribosome. The failure of cell-free heterologous systems to cleave preproteins when membranes are added after translation could be due to factors such as altered folding of the polypeptide or inaccessibility of a membrane-bound protease. Resolution of this question requires further knowledge of the details of the transport and cleavage of secretory proteins.

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