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THE CELL FREE SYNTHESIS OF BOVINE LUTROPIN β SUBUNIT*

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SUMMARY

RNA prepared from bovine pituitary glands directs the synthesis of trichloroacetic acid-insoluble proteins in the wheat germ cell-free system. Antisera specific to bovine lutropin (LH) beta subunit precipitated a product with a molecular weight of approximately 17,000, suggesting a precursor. Its specificity was demonstrated by the failure of antisera to other pituitary hormones, including LH alpha and thyrotropin (TSH) beta, to successfully precipitate it and also by its competition with unlabeled LH β for the beta antisera. The addition of microsomal membranes resulted in the partial processing of this 17,000 dalton protein to a product of higher molecular weight (Mr~19,000), suggesting glycosylation.

The cell-free synthesis of the beta subunit of the glycoprotein hormones has been difficult to demonstrate. Although previous studies using mRNA from first trimester placentae and mouse thyrotropic tumors have identified the synthesis of a precursor form of the alpha subunit of hCG[1] and TSH (1-5), demonstration of beta was achieved only after further purification of the mRNA and by optimizing magnesium concentrations in the cell-free assay.

Despite these studies there has been no evidence for the synthesis of the gonadotropin beta subunit in the normal pituitary gland. Since it has been suggested that the synthesis of beta may be rate limiting in the production of the intact hormones (1-3, 6-11), the demonstration of the synthesis of the beta subunit is crucial to the study of the mechanisms of biosynthesis of these glycoprotein hormones.

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[1] The abreviations used are : hCG, human choriogonadotropin; TSH, thyrotropin; LH, lutropin; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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In this study, we have demonstrated the in vitro synthesis of the LH beta subunit using bovine pituitary RNA in a heterologous cell-free system and shown that microsomal membrane addition to this cell-free system results in the partial processing of this product to a larger molecular weight protein ($M_r \sim 19,000$) which contained carbohydrate. The evidence presented suggests that the beta subunit of bLH is synthesized as a precursor which can be glycosylated in the presence of membranes.

MATERIALS AND METHODS

RNA Preparation - RNA was extracted from steer pituitary glands as described previously (12), and obtained in yields of approximately 1 mg/g of pituitary tissue.

Cell Free Assays - The wheat germ S-30 extract was prepared as described by Roberts and Paterson (13) with omission of the preincubation step. The concentrations of the reagents in the assay were 15 mM Hepes (N-2-hydroxyethyl-1-piperazineethanesulfonic acid) (pH 7.2), 0.3 mM GTP, 1.5mM ATP, 40 ug/ml of creatine kinase, 8mM phosphocreatine, 72mM KCl, 2mM magnesium acetate, 40uM concentration each of the amino acids except methionine, 400 uM spermidine, 2.5mM dithiothreitol, 4uCi [^{35}S]methionine, 10ug RNA and 10 ul S-30.

Product Analyses - Cell-free products were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoprecipitation as previously described (12).

Autoradiography - Gel slabs were dried and exposed to Kodak XR-5 X-omat film. Fluorography was performed according to Bonner and Laskey (14).

Glycosidase Modifications - Glycosidase treatment of the immunoprecipitates was performed as described by Bielinska and Boime (1978).

Reagents - All chemicals were reagent grade including the phenol which was redistilled and saturated with buffer (Tris- HCl, pH 8.5) prior to use. The [^{35}S]methionine (800 to 1200 Ci/mmol) was obtained from Amersham. The purified bovine subunits and their specific antisera were generously supplied by John Pierce. Ascites microsomal membranes were a gift from Irving Boime. α-Mannosidase (E.C.3.2.1.24) was obtained from Boehringer-Mannheim. All glassware was autoclaved.

RESULTS

The translation of pituitary RNA in the wheat germ cell-free system resulted in the stimulation of [^{35}S]methionine incorporation into TCA-insoluble protein approximately 5-fold over background. The fidelity of translation of this RNA was demonstrated by the synthesis of

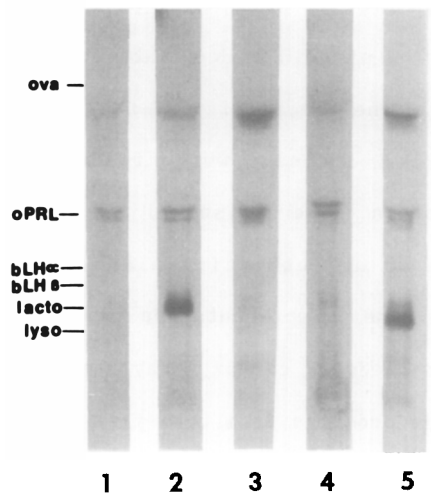

Figure 1. Fluorograph of ^{35}S-labeled protein synthesized in a wheat germ system programmed with bovine pituitary RNA. Translation reactions and electrophoresis conditions are described under Experimental Procedures. Following translation the reaction mixtures were centrifuged at 150,000 x g for 1 h and then subjected to immunoassay. Lane 1, normal rabbit serum; Lane 2, bLH ß antisera; Lane 3, bTSH ß antisera; Lane 4, bLHß antisera plus 50 µg of unlabeled, purified bLHß; and Lane 5, reduced carboxymethylated bLH α antisera. The amount of radioactivity applied to the gels was 4000-5000 cpm and fluorography was for 10 days. The standard proteins were: ova (ovalbumin, 43,000); oPRL (ovine prolactin, 22,000); lacto (lactoglobulin, 18,400) and lyso (lysozyme, 14,300). The apparent molecular weights of bLH alpha (bLHα) and beta (bLHß) on SDS-PAGE are approximately 22,000 and 19,000, respectively, which differs from the chemical determinations of 12,000 and 14,000 (21). This anomolous migration is due to the presence of carbohydrate.

two major proteins which were identified as preprolactin and pregrowth hormone by immunoprecipitation (12).

The identification of LH beta was achieved using highly specific LH ß antisera and is shown in Fig. 1. Lane 1 represents cell-free products immunoprecipitated with normal rabbit serum. As can be seen, some nonspecific products (molecular weights, ∿ 25,000 and 40,000) were precipitated with this nonimmune sera. As indicated previously (12), these products, when observed, demonstrated no specificity and were present in all samples, including those which had no RNA added, regardless of antisera. These labeled products were associated with the two major stained proteins which may represent the dissociated light and

heavy immunoglobulin chains. When the products were reacted with specific LH β antisera, a protein with a molecular weight of 17-18,000 was apparent (Lane 2). The specificity of this product was evidenced by: 1) the use of TSH β antisera which failed to demonstrate any immunoprecipitable protein (Lane 3), and 2) the successful competition of this product with 50 ug authentic unlabeled bLH β for the bLH β antisera (Lane 4). Lane 5 represents the immunoprecipitation of the cell-free products with reduced carboxymethylated bovine LH α antisera (12). In addition, the products from cell-free assays which contained either no pituitary RNA or globin RNA failed to demonstrate an immunoprecipitable band in the presence of the beta antisera (data not shown).

It is evident from these gels that a protein with a molecular weight of 17-18,000 contains immunological determinants similar to bovine LH β, although its size is slightly larger than the protein portion of the authentic beta subunit ($\sim$ 14,000), suggesting the possibility of a precursor.

When products of the cell-free system programmed with pituitary RNA and treated with 1 ul of ascites membranes per reaction mix were immunoprecipitated with bLH β antisera, a slight diminution of the 17-18,000 dalton product was observed (Fig. 2; Lane 3 vs Lane 2). Concomitant with this was the appearance of a specific immunoprecipitated product demonstrating a molecular weight of approximately 19,000. Both of these products successfully competed for the specific bLH β antisera (Lane 4) while neither were present when normal rabbit serum (Lane 1) or other specific pituitary alpha antisera were used (Lanes 5-6). The difference between the specifically immunoprecipitated alpha products in lanes 5 and 6 is the absence (Lane 5) and presence (Lane 6) of membranes.

Since it has been demonstrated that membrane dependent glycosylation occurs in cell-free systems (15-20), and since the

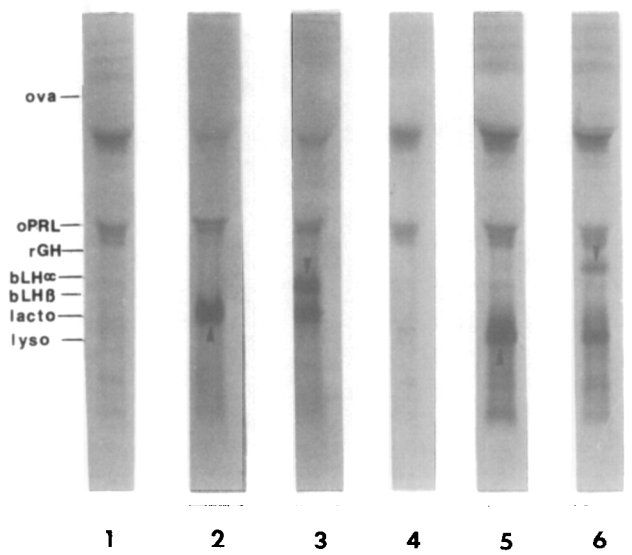

Figure 2. Fluorograph of ^{35}S-labeled proteins synthesized in response to bovine pituitary RNA in a wheat germ system containing 1 µl of ascites microsomal membranes per reaction mix. After removal of the ribosomes, the reaction mixtures were subjected to immunoprecipitation with specific antisera as previously described (12). Lane 1, normal rabbit serum; Lane 2, bLHß antisera, no membranes; Lane 3, bLHß antisera plus membranes; Lane 4, bLHß antisera plus membranes, plus 50 µg unlabeled bLHß; Lane 5, bLHα antisera, no membranes; and Lane 6, bLHα antisera plus membranes. Approximately 3500 cpm were added to each lane and fluorography was for 14 days. The standards are defined in the legend for Figure 1. The arrowheads which point upward indicate the products synthesized in the absence of membranes, whereas those arrowheads pointing downward indicate the products which appeared following the addition of membranes.

migration of this product is almost identical to that of the mature beta subunit (Fig 2, bLHß), the products were they examined for the presence of carbohydrate. Immunoprecipitated products synthesized in the presence of membranes were treated with α-mannosidase (15,16) and examined on SDS-PAGE (Fig 3). The 19,000 dalton product which had been precipitated with beta antisera following membrane addition (Lane 3) disappeared as a result of treatment with α-mannosidase (Lane 4). Concomitant with the loss of this product was the appearance of a heterogeneous product with a molecular weight range of 16,000-18,000 daltons (Lane 4). It was suspected that the glycosidase treatment resulted in a population of beta molecules with varying amounts of

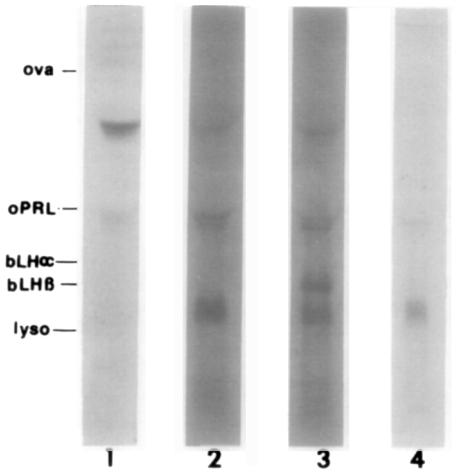

Figure 3. Fluorograph of ^{35}S-labeled proteins synthesized in response to bovine pituitary RNA in wheat germ system containing 1 µl of ascites microsomal membranes per reaction mix. Following immunoprecipitation, the precipitates were treated with α-mannosidase (15). The enzyme-treated products were dissolved in electrophoresis sample buffer (12) and applied to SDS-PAGE. Lane 1, normal rabbit serum; Lane 2, bovine LH β antisera, no membranes; Lane 3, bovine LHβ antisera plus membranes; and Lane 4, bovine LHβ antisera plus membranes plus α-mannosidase. Approximately 4000 cpm were applied and the gels exposed to fluorography for 12 days.

carbohydrate. As a control, treatment of the products in Lane 2 with this enzyme had no effect on their migration.

DISCUSSION

We have demonstrated the synthesis of the LH beta subunit in a heterologous cell-free system programmed with bovine pituitary RNA. This protein was identified by immunoprecipitation with specific bLH β antiserum. Its immunological specificity was demonstrated by the failure of bTSH β, bLH α, ovine prolactin and rat growth hormone antisera to immunoprecipitate this product and by its competition with authentic bLH β for the antisera. When this product was examined on SDS-PAGE, it exhibited a molecular weight of 17-18000 which is approximately 3000 daltons larger than the protein portion of LH β (21) suggesting a precursor as has been reported for hCG β (3) and TSH β (4).

The addition of microsomal membranes to the cell-free system resulted in the appearance of an immunoprecipitable product with a molecular weight of approximately 19,000. This protein appeared to be the result of at least a partial processing of the 17000 dalton-product as its appearance was dependent upon membrane addition. Based on the migration of this product in SDS-PAGE and the shift in its migration as a result of mannosidase treatment, it was suggested that the protein was glycosylated as a result of membrane addition.

These results represent the first reported evidence for the *in vitro* synthesis of a gonadotropin beta subunit translated by RNA from the normal pituitary gland. This evidence, in combination with similar findings regarding the alpha subunit, strongly suggest that 1) the alpha and beta subunits of pituitary LH are synthesized separately, 2) the subunits are synthesized as precursors and 3) the subunits are glycosylated in the presence of microsomal membranes, presumably while still on the ribosome.

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