PRECURSOR–PRODUCT RELATIONSHIPS IN THE BIOSYNTHESIS AND SECRETION OF THYROTROPIN AND ITS SUBUNITS BY MOUSE THYROTROPIC TUMOR CELLS

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1. Introduction

TSH is composed of two non-covalently linked, glycosylated subunits, an α subunit that is virtually identical to that in LH, FSH, and CG as well as a unique β subunit that confers biologic specificity [1]. Although heterogeneous molecular weight forms of TSH as well as other glycoprotein hormones and their subunits have been recognized [2–6], the precursor–product relationships among various forms have not yet been elucidated. In this report we describe such relationships in the de novo biosynthesis of TSH using primary cultures of dispersed mouse pituitary thyrotropic tumor cells.

2. Materials and methods

Pituitary thyrotropic tumors (NIH 102) were induced and transplanted as described previously [4,7]. Cells were enzymatically dispersed by the method of Vale et al. [8] and suspended in Dulbecco's Modified Eagle's Medium supplemented with 10% hypothyroid calf serum (Rockland Farms), 10 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. One ml of cell suspension (approx. 10^6 cells) was added to 1.5 ml of cell-free medium in 25 cm^2 culture dishes (Falcon Plastics) and incubated at 37°C in a moist atmosphere of 5% CO_2–95% air until confluency was reached at 6 days. Culture dishes were washed 4 times with 2 ml methionine-free medium; 2.5 ml of the medium containing 100 μCi [^{35}S] methionine (Amersham/Searle, 1175 Ci/mmol) was then added to each dish. In continuous labeling experiments the dishes were incubated with [^{35}S]methionine for 24 h. In 'pulse-chase' experiments dishes were incubated with the labeled amino acid for 10 min, after which further de novo incorporation was inhibited by the addition of 10 000-fold excess of unlabeled methionine. After replacement of medium with an equivalent volume containing 0.7 mM methionine, incubation was continued for 0, 60, 120 or 240 min. At the end of the labeling period media were decanted, cell lysates prepared [4], and both were centrifuged at 1000 × g for 10 min. In addition, lysates were centrifuged at 100 000 × g for 2 h.

Replicate samples for each time point were pooled and dialyzed extensively at 4°C against a solution of 0.01 M sodium phosphate, 0.15 M sodium chloride, 0.02% (w/v) sodium azide, pH 7.4 (PBS-azide). Samples were concentrated by ultrafiltration (UM-10 membrane, Amicon) to a volume of 1.5 ml and precipitation was performed [9] by the addition of 10 μl of nonimmune guinea-pig serum (Pentex) and 50 μl of sheep anti-guinea pig gamma globulin. After incubation for 16 h at 4°C samples were centrifuged at 1000 × g for 20 min and the supernatant applied to a Sephadex G-100 (Pharmacia) column (90 × 1.5 cm) equilibrated at 4°C with PBS-azide containing 0.1% (w/v) ovalbumin (Sigma). The column was eluted at a flow rate of 4 ml/h and 2 ml fractions were collected.

Triplicate aliquots (50–200 μl) of gel chromatog-

Abbreviations: b, bovine; CG, chorionic gonadotropin; FSH, follicle-stimulating hormone; h, human; LH, luteinizing hormone; PBS, phosphate-buffered saline; r, rat; SDS, sodium dodecyl sulfate; TSH, thyrotropin

raphy fractions were incubated in 1 ml of a solution containing PBS, 7 mM methionine, and 0.5% (v/v) Nonidet (Shell). To each of the triplicate samples was added either 3.5 μl nonimmune rabbit serum, 2 μl rabbit anti-bovine TSH-α or 3 μl anti-bovine TSH; these amounts of antisera, which had been generated against purified bovine materials generously supplied by Dr John Pierce, contained identical amounts of total gamma globulin. Samples were incubated for 2 h at 25°C, 30 min at 4°C and then 75 μl sheep anti-rabbit gamma globulin was added. After further incubation for 18 h at 4°C the precipitates were centrifuged at 2000 × *g* for 60 min through 1 M sucrose, 0.5% (v/v) Nonidet using the technique of Rhoads et al. [10]. Precipitates were solubilized in 1 ml Protosol (New England Nuclear) at 37°C for 15 min and transferred to scintillation vials. After the addition of 10 ml Econofluor (New England Nuclear) samples were counted in a scintillation counter at an efficiency of 72%. Other immunoprecipitates were dissolved in a solution containing 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol at 90°C for 5 min and analyzed by electrophoresis on 10% polyacrylamide slab gels containing 0.2% SDS using previously published methods [11]. Two mm gel slices were added to scintillation vials, extracted (approx. 70% recovery of radioactivity) in 1 ml Protosol, 15 ml Econofluor for 16 h at 37°C and then counted.

3. Results

Figure 1 depicts the immunoprecipitation of intracellular and extracellular [^{35}S]methionine radioactivity from continuous 24-h labeling experiments after fractionation on Sephadex G-100. Under these conditions the anti-α serum employed had been previously shown to precipitate over 85% of purified labelled rLH-α or rTSH but less than 10% of rTSH-β; the anti-TSH serum had precipitated over 85% of purified labeled rTSH-β or rTSH but less than 20% of rLH-α. Intracellular and extracellular radioactivity in the V_0 region of the G-100 column showed a large amount of nonspecific precipitation by nonimmune rabbit serum but no additional specific precipitation with anti-α or anti-TSH serum.

The predominant intracellular component specifically precipitated was TSH (fig.1, effluent volume 70–80 ml) which was identified by its elution position similar to standard rTSH, its nearly equal precipitation by anti-α and anti-TSH sera, and its dissociation into α and β subunits in SDS gel electrophoresis (see below). Another major intracellular component was free α (fig.1, effluent volume 85–90 ml), identified by its elution position similar to standard rLH-α, its excess precipitation by anti-α compared to anti-TSH

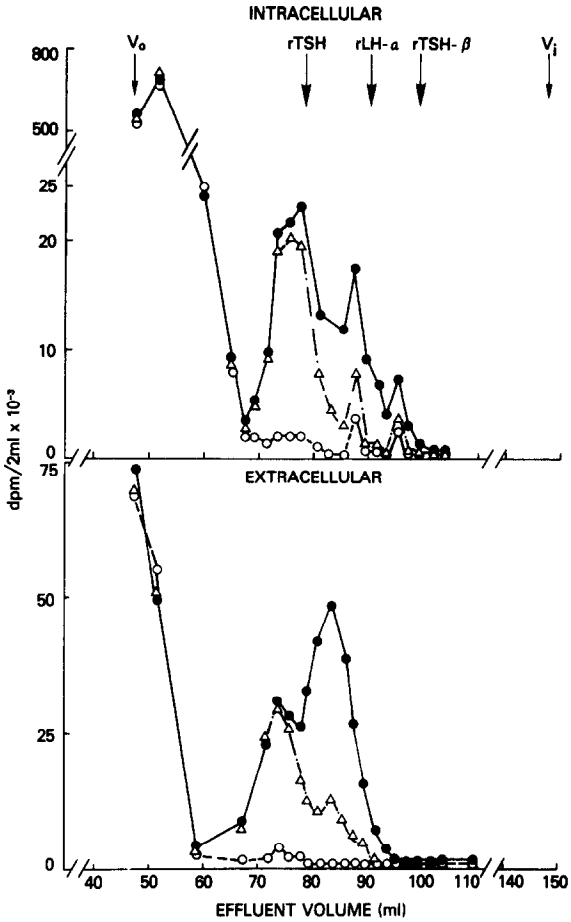

Fig.1. Sephadex G-100 chromatography of dispersed thyrotropic tumor cell lysate (intracellular) and medium (extracellular) after continuous 24-h labeling with [^{35}S]methionine. Samples were chromatographed and labeled proteins in each column fraction were precipitated with rabbit nonimmune serum (o--o), anti-bTSH-α (•—•), or anti-bTSH (△—· △) as described in section 2. At the top of the figure are indicated the column void volume (V_0) and internal volume (V_i) as well as the elution positions of standard rTSH, rLH-α, and rTSH-β.

serum, and its behavior in SDS gel electrophoresis. Smaller amounts of lower molecular weight α forms were also noted intracellularly (fig.1, effluent volume 90–100 ml). However no radioactivity eluting similar to standard free rTSH-β and demonstrating excess precipitation by anti-TSH compared to anti-α was demonstrated.

In contrast to the intracellular radioactivity, the extracellular immunoreactive material (fig.1) was predominantly free α with relatively less TSH. Moreover both the extracellular TSH and free α had slightly lower elution volumes and, thus, higher apparent molecular weights than the respective intracellular forms or the rat standard glycoproteins. Neither lower molecular weight α forms nor any form of TSH-β was identified extracellularly.

The nature of the 24-h labeled material from the G-100 column was further characterized by SDS gel electrophoresis under conditions of dissociation and reduction (fig.2). Intracellular material from the V_0 region of the column revealed only large molecular weight components (predominantly > 100 K and 55 K) and no additional specific precipitation by anti-α compared to nonimmune serum. Intracellular (data not shown) and extracellular material from the TSH region of the column that was specifically precipitated by either anti-α or anti-TSH dissociated into two subunits (20–21 K and 18–19 K) similar to standard rLH-α and rTSH-β. The extracellular material from the α region of the column that was specifically precipitated by anti-α was similar to the α subunit dissociated from TSH. Intracellular material from the α region of the column also showed a similar predominant α form (20–21 K) but also a small amount of lower molecular weight (14–17 K) α forms.

The precursor–product relationships of these various intracellular and extracellular TSH and subunit forms were examined by 'pulse-chase' experiments (fig.3). After a 10 min pulse of [^{35}S]methionine the predominant intracellular component was a lower molecular weight α form similar to the minor component in 24-h labeling studies, and no TSH or TSH-β was observed. When the 10-min pulse was followed by a 1–4 h chase of excess unlabeled methionine the α progressively shifted to higher molecular weight forms and TSH first appeared at 1 h. In recent experiments TSH subunit combination was shown to occur between 10 and 30 min after the pulse period. Extracellular

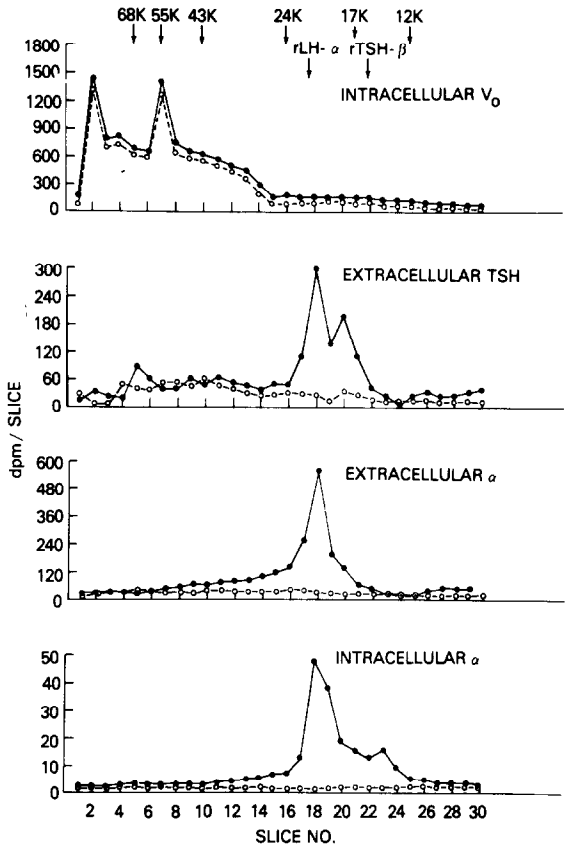

Fig.2. SDS polyacrylamide slab gel electrophoresis of ^{35}S radioactivity precipitated by rabbit nonimmune serum (o--o) or anti-bTSH-α (•—•) in Sephadex G-100 column fractions (see fig.1 and section 2). The individual figures represent immunoprecipitates of intracellular V_0 proteins (fig.1, elution volume 46–47 ml), extracellular TSH (69–70 ml), extracellular α (83–84 ml) and intracellular α (95–100 ml). At the top of the figures are indicated the positions of albumin (68 K), IgG heavy chain (55 K), ovalbumin (43 K), IgG light chain (24 K), myoglobin (17 K), and cytochrome *c* (12 K) standards as well as rLH-α (20–21 K) and rTSH-β (16–17 K).

TSH and α appeared between 1–2 h and by 4 h most of the labeled material was secreted. As had been noted in 24 h labeling experiments, there was a preferential secretion of free α, and media TSH and α had slightly higher apparent molecular weights than the respective intracellular forms. No free TSH-β was identified either in cells or in media at any time.

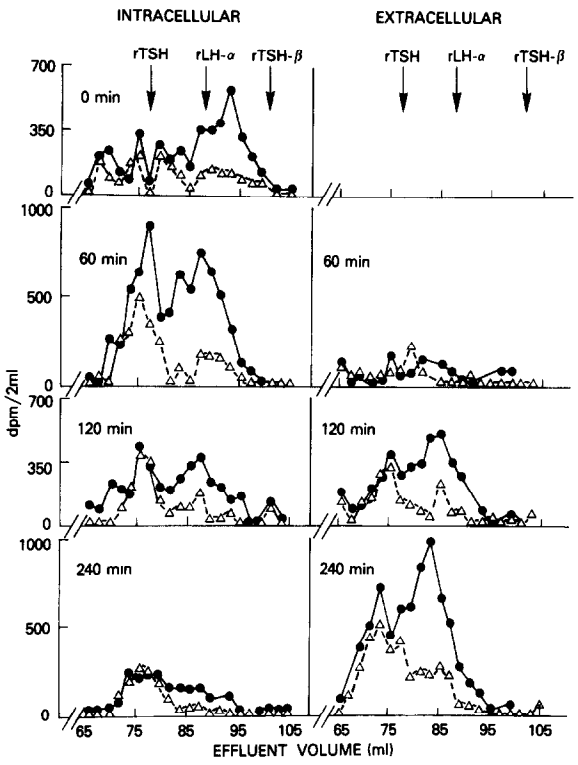

Fig.3. Sephadex G-100 chromatography of cell lysate (intracellular) and medium (extracellular) from a 'pulse-chase' experiment. Cells had been exposed to a 10 min pulse of [^{35}S]methionine followed by a chase of 10 000-fold excess unlabeled methionine for the times indicated. Samples were chromatographed and the radioactivity in column fractions precipitated with rabbit anti-bTSH-α (●—●) or anti-bTSH (△—△) as described in section 2. The small amount of radioactivity precipitated by nonimmune serum (not shown) was subtracted from that precipitated by each antiserum.

4. Discussion

We have previously suggested that the α and β subunits of TSH and other structurally related glycoprotein hormones are synthesized on separate polyribosomes and later combine intracellularly to form the active hormone [4,5,12]. This hypothesis was primarily based on our recognition of many instances of isolated or highly unbalanced production of α or β subunits both in vivo and in vitro [4,5], as well as the demonstration by several groups of spontaneous subunit combination in vitro [1,12]. With the discovery of large immunoactive forms of glycoprotein hormones [2,3,6], certain other investigators have suggested that the α and β subunits are initially covalently linked in a precursor protein, analogous to the linkage of the A and B chains of insulin by the connecting peptide in proinsulin [6]. However, recent studies of the cell-free translation of the messenger RNA for hCG [13,14] and TSH [15,16] have suggested separate synthesis of α and β subunits.

We have previously demonstrated that dispersed mouse thyrotropic tumor cells secrete heterogeneous forms of unlabeled TSH and excess free α subunit but no gonadotropin or prolactin [4]. However, the precursor–product relationships among these subunit and hormone forms could not be determined prior to the current studies employing 'pulse-chase' analysis of labeled amino acid incorporation [17]. Although such methods may not identify initial 'pre-subunit signal' forms [13,15,16,18] which may be processed even before completion of the nascent polypeptide chains, they should have identified any prohormone forms similar to proinsulin [17] or proparathyroid hormone [19] which are processed after chain completion and release from the polyribosome. Our failure to detect pro-TSH or prosubunits suggests that glycoprotein hormone biosynthesis, like that of prolactin, growth hormone, and placental lactogen [9] may not require prohormone intermediates.

In the current studies newly labeled TSH and free α were identified by their relative reactivity with two specific antisera directed at different subunit determinants [4], as well as by their behavior in gel chromatography under non-dissociating conditions and in SDS gel electrophoresis under conditions of dissociation and reduction. However, we also observed relatively large amounts of high molecular weight proteins that nonspecifically precipitated with nonimmune serum but showed no additional specific precipitation with the two antisera. These large proteins, which showed no pulse-chase relationships to TSH and were not observed in cell-free translation of messenger RNA from these tumors [15,16], have been identified recently as collagen components arising from fibroblast contamination of these primary monolayer cultures [20]. However, the presence of these co-precipitating large proteins makes it difficult for us to entirely exclude the possibility of additional high molecular weight (> 100 000 daltons) TSH components.

These data suggest that a low molecular weight α form is initially synthesized before any free β or TSH appears. TSH subunit combination occurs soon after the conversion to higher molecular weight α forms and the appearance of the limiting β subunit. The latter is immediately combined with excess 'mature' α subunit and is not detected in a free β form. Secretion of TSH and excess free α is delayed and restricted to the highest molecular weight forms, as has been noted in vivo [21]. Preliminary experiments using [^{3}H]glucosamine as a precursor suggest that the conversion from lower to higher molecular weight α and TSH forms reflects progressive glycosylation. However, direct amino acid and carbohydrate analyses will be required to elucidate the precise chemical nature of these various forms.

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