Evidence for Conformational Differences between Precursor and Processed Forms of Thyroid-stimulating Hormone β Subunit*

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Linda C. Giudice and Bruce D. Weintraub
From the Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20205

mRNA from a mouse pituitary thyrotropic tumor was translated in a wheat germ extract containing [35S]Met and coded for a major protein (M, = 17,000), previously identified as a precursor of the α subunit, "pre-α," of thyroid-stimulating hormone (TSH), and a less abundant protein of M, = 15,500. The latter, which is about 2500 daltons larger than the protein moiety of TSHβ, was specifically immunoprecipitated from translations with antisera against denatured but not native TSHβ and is believed to be a precursor of the β subunit, "pre-TSHβ." At least two tryptic peptides of pre-TSHα and putative pre-TSHβ coincided with peptides from authentic bovine α and TSHβ, respectively. When thyrotropic tumor microsomal membranes were added during translation of tumor mRNA, a new major (M, = 22,000) and minor (M, = 18,500) protein were observed. The former was specifically immunoprecipitated with anti-α serum and, in gel electrophoresis, co-migrated with authentic TSHα. The 18,500-dalton protein was specifically immunoprecipitated by antisera to native but not denatured TSHβ and co-migrated with authentic TSHβ. TSH, secreted by thyrotropic tumor cells in culture, was recognized by antisera against native α and β, but not by the antisera to denatured TSHβ.

The data show that TSHβ, as well as the previously identified α subunit, is synthesized as a precursor, "pre-β," supporting the thesis that the subunits are synthesized from separate mRNAs. Also, co-translational addition of homologous microsomal membranes converts the pre-subunits to forms that are, by gel electrophoresis, identical to glycosylated, secreted TSHα and β. Furthermore, the differential reactivity of pre-TSHβ with antisera to denatured and native TSHβ suggests that the combination of pre-TSHβ differs from that of the subunit, processed by microsomal membranes or in combination with TSHα in the intact hormone.

Thyroid-stimulating hormone, like the other pituitary glycoprotein hormones and the homologous placental protein, choriogonadotropin, consists of two, noncovalently associated subunits, α and β. The α subunits are common among the hormones, whereas the β subunits are unique and confer hormonal specificity to each dimer (2, 3). Because normal pituitary contains low levels of the hormones, biosynthesis of these complex, secreted proteins has been studied in human placenta (4-9) and a mouse thyrotropic tumor (10-14), tissues whose major biosynthetic products include, respectively, hCGα and TSH. In cell-free systems a precursor of hCGβ was first reported by Landefeld et al. (4), but detection of a precursor of hCGβ proved more difficult, in part because the placenta, like the pituitary, synthesizes significantly more α than β subunits (15-17). Pre-hCGβ was subsequently identified immunochemically with an antisera to denatured, mature hCGβ (6). Precursor forms of both subunits are consistent with the "signal hypothesis" for secreted proteins (18, 19), and partial processing of pre-hCGα by heterologous microsomal membranes recently has been demonstrated (5, 7).

mRNA from a mouse pituitary thyrotropic tumor, which synthesizes primarily TSH and its α subunit (10, 11), also has been translated in several cell-free systems (12-14). A tumor protein about 3000 daltons larger than nonglycosylated TSHα and having tryptic peptides in common with authentic TSHα was tentatively identified as pre-TSHα (12). In reports from this laboratory, pre-TSHα was identified immunochemically in translations of mouse tumor mRNA (13, 14), and we have shown it is identical in size to normal mouse pituitary pre-α (14). Further, mouse pre-α has a signal peptide of at least 29 residues, which is homologous with normal pituitary and also placental pre-α subunits (14). While identification of pre-TSHα is now well established, definitive identification of a precursor of TSHβ has been lacking. In cell-free translations a tumor protein (M, = 17,000), larger than core, nonglycosylated TSHβ (M, = 13,000), was postulated as pre-TSHβ (12); however, no direct evidence showed a relationship between this protein and TSHβ. In the present communication we report the synthesis, specific immunoprecipitation, and homologous processing of "pre-TSHβ" (M, = 15,500). The relative conformational states of pre-TSHβ and its corresponding membrane-processed form are discussed in the context of why immunological detection of β synthesis in cell-free systems has been enigmatic.

EXPERIMENTAL PROCEDURES

Most of the procedures used have been described elsewhere. Among these are: pituitary thyrotropic tumor induction and transplantation (10); RNA extraction, mRNA isolation, wheat germ extract preparation and in vitro translations, and assessing incorporation of radiolabeled amino acids into translated proteins (14). Translations containing 130 mM K+, 2 mM Mg++, and 66 μg/ml of mouse tumor mRNA were incubated for 1 h at 27°C (14). Rabbit reticulocyte lysates (20) were preincubated with micrococcal nuclease (21), and translation mixtures were incubated for 1 h at 29°C (22). A total microsomal fraction was prepared from freshly excised mouse thyrotropic tumors according to Katz et al. (23), and endogenous ribosomes

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† Recipient of National Research Service Award 5332 AM05790-02. Present address, Dept. of Neurobiology and School of Medicine, Stanford University, Stanford, Calif. 94305.

† The abbreviations used are: h, human; CG, choriogonadotropin; TSH, thyrotrophic-stimulating hormone; L11, lutetinizing hormone; FSH, follicle-stimulating hormone; b, bovine; SDS, sodium dodecyl sulfate; M, apparent molecular weight; EDTA, (ethylenedinitrilo)tetraacetic acid; RCM, reduced, carboxymethyl-.
were "stripped" with EDTA (23). Stripped microsomes were added to reticulocyte lysate translations at a final concentration of 1.6 A<sub>280</sub> units/ml in wheat germ extracts, microsomes isolated protein synthesis.

Anti-bovine α serum ("anti-α") was generated at the National Institutes of Health by immunizing rabbits with native bLHa, provided by Dr. J. G. Pierce, University of California, Los Angeles. Antiserum to native bTSHβ ("anti-TSHβ") was supplied by Dr. I. A. Kourides of Memorial Sloan-Kettering Cancer Institute. Under conditions of antibody excess, anti-TSHβ immunoprecipitated about 85% of isolated, <sup>35</sup>S-labeled rat TSHβ and less than 1% of <sup>35</sup>S-labeled rat α subunit; since purified mouse α and TSHβ are not available, purified rat subunits (shown to be similar to the mouse subunits (10)) were used in these experiments. Immunoprecipitation with bTSHα subunits and an antiserum raised against reduced, carboxymethyl-bTSHβ (24) ("anti-RCM TSHβ") did not immunoprecipitate secreted TSHβ at any dilution examined (1:10 to 1:10,000). Antigen-antibody complexes were precipitated with Cowan 1 strain of Staphylococcus aureus (Calbiochem) (26).

Translated proteins were analyzed (14, 22) by electrophoresis in SDS-gradient polyacrylamide slab gels (15.5 mm thick) having a 5% polyacrylamide stacking gel (1 or 2 cm) and a 12 to 20% polyacrylamide gradient resolving gel (8.7 or 26 cm) (14). After electrophoresis gels were stained, destained, and prepared for fluorography (27). Dried gels were exposed to Kodak X-Omat XR-2 film for various periods of time, and films were developed by conventional procedures.

In peptide mapping procedures immunoprecipitated, labeled mouse presubunits (from a 1-ml translation) or authentic (unlabeled, bovine) subunits (0.75 mg) were boiled for 3 min in 200 μl of 50 mM Tris-HCl, pH 8.8, buffer containing 8 M urea and 0.1 M dithiothreitol. After cooling to 25°C, 200 μl of 0.5 M iodoacetamide were added and mixtures were incubated at 25°C for 45 min in the dark. Subunits were mixed (pre-α with bLHa, and pre-β with bTSHβ), dialyzed against 0.125 M NH₄HCO₃, lyophilized, redissolved in 160 μl of 0.126 M NH₄HCO₃, and digested with trypsin (28). Resulting peptides were separated by descending chromatography on Whatman 3MM paper in the upper phase of 1-butanol/acetic acid/water (4:1.5, v/v) for 16 h, followed by electrophoresis at right angles (2000 V, 1 h) in 0.6 M pyridine/acetic acid, pH 3.6, buffer. Dried maps were stained with 0.1% ninhydrin in 95% ethanol containing 5% collidine. Autoradiographs of dried, stained maps were effected with Kodak X-Omat films after exposure to radiolabeled samples.

**RESULTS**

Analysis of translated proteins (Fig. 1) revealed one major protein (Lane 1, M<sub>e</sub> = 17,000) that resolved into major (M<sub>e</sub> = 17,000) and minor (M<sub>e</sub> = 15,500) proteins on a longer gel (Lane 2). The dominant band was immunoprecipitated with anti-α serum (Lane 3) and previously was shown to be a precursor of TSHα, pre-α (14). Anti-TSHβ did not immunoprecipitate any translated proteins (Lane 4). However, antisera to denatured, reduced, carboxymethyl-TSHβ (anti-RCM TSHβ) immunoprecipitated a protein (Lane 6) that apparently co-migrated with the 15,500-dalton minor band (Lane 2) and probably is a precursor of the 13,000-dalton (nonglycosylated) β subunit (pre-TSHβ). Some cross-reactivity between anti-RCM TSHβ and the large excess of pre-α (Lane 6, M<sub>e</sub> = 17,000) was observed to homologous regions of primary structure in α and TSHβ (30). A minor 14,000-dalton protein also immunoprecipitated may be an early termination form of pre-α or -β, or both (see legend to Fig. 1). Specificity of pre-TSHβ immunoprecipitation was shown in competition experiments (Lanes 7 and 8) in which TSHβ but not TSHα competed with putative pre-β for anti-RCM TSHβ. Since little or no cross-reactivity was observed with anti-RCM TSHβ and native TSHβ (24), competition of native with pre-β (Lane 7) most likely is due to the intentional excess of unlabeled antigen. An equivalent excess of authentic α, however, did not compete with pre-TSHβ for anti-RCM TSHβ (Lane 8).

Partial chemical characterization of the presubunits was achieved by peptide mapping analyses. Fig. 2 shows maps of trypsin-digested bLHa and <sup>35</sup>S-labeled pre-α (Panels A and B), and also bTSHβ and pre-TSHβ (Panels C and D). Two major labeled peptides in pre-α coincided precisely with two major ninhydrin-staining peptides from bovine α (cf. Panels A and B). Considering conservation of Met in α subunits from various species (2, 3), at most two or three Met-containing peptides would be expected to overlap. These data along with microsequencing data of the signal peptide of pre-α (14) support the identity of the 17,000-dalton α-immunoreactive protein as pre-TSHα. Two <sup>35</sup>S-labeled peptides in putative pre-β (Panel D) coincided precisely with two ninhydrin-staining peptides from bTSHβ (Panel C); at most, two Met-containing peptides would be expected to overlap considering the differences in species (2, 3). The maps of labeled pre-α and -β clearly differ, and the data, taken together, support the identity of the 15,500-dalton β-immunoreactive protein as pre-TSHβ.

The relative amounts of pre-TSHβ subunits immunoprecipitated from translation mixtures are shown in Table I. Anti-α immunoprecipitated about 25% of the trichloroacetic acid-insoluble radiolabel, and anti-RCM TSHβ immunoprecipitated about 5%. Anti-TSHβ and nonimmune serum immunoprecipitated about equal amounts of labeled material (cf. Fig. 1, Lanes 4 and 9). Specificity of immunoprecipitations is also in Table I.
When stripped microsomes from the thyrotropic tumor were added during translation of tumor mRNA, two new proteins were observed (Fig. 3, cf. Lanes 4 and 7) whose molecular weights corresponded to mature $\alpha$ ($M_\alpha = 22,000$) and $\beta$ ($M_\beta = 18,500$). Some pre-$\alpha$ ($M_\alpha = 17,000$) also was observed, indicating incomplete processing by microsomal membranes, and the large mass of globin in the reticulocyte lysate probably obscured any unprocessed pre-TSH$\beta$. Nonetheless, anti-$\alpha$ immunoprecipitated a membrane-processed protein ($M_\alpha = 22,000$, Lane 5) that co-migrated with authentic TSH$\alpha$ (Lane 2); bTSH$\alpha$ competed with this protein for anti-$\alpha$ serum (Lane 6). Anti-RCM TSH$\beta$ serum did not immunoprecipitate any membrane-processed proteins (Fig. 4A, Lane 5; Fig. 4B, Lane 2) at any dilution examined (1:10 to 1:10,000). Anti-TSH$\beta$, however, immunoprecipitated predominantly a membrane-processed protein ($M_\beta = 18,500$; Fig. 4A, Lane 5; Fig. 4B, Lane 3) which competed with excess authentic bTSH$\beta$ for anti-TSH$\beta$ (Fig. 4B, Lane 4). Some co-precipitation of excess pre- and processed $\alpha$ was also observed (Fig. 4B, Lane 6).

### CHROMATOGRAPHY

**Fig. 2.** Comparison of peptides of authentic bovine subunits and mouse pre-$\alpha$ and $\beta$. $[^{35}S]$Met-labeled pre-TSH$\alpha$ and $\beta$, respectively, were mixed with authentic bL$\alpha$ and bTSH$\beta$, digested with trypsin, and fractionated by two-dimensional paper chromatography and electrophoresis. A, ninhydrin-stained map of bL$\alpha$ and labeled pre-$\alpha$. B, autoradiograph of map in A. C, ninhydrin-stained map of bTSH$\beta$ and labeled pre-$\beta$. D, autoradiograph of map in C. Arrows indicate coincident peptides. Maps of bL$\alpha$ and bTSH$\beta$ are similar to those previously reported using a different solvent system (31). The identities of bovine peptides on maps, however, have not been determined (31).

### ELECTROPHORESIS

**Fig. 3.** Fluorographic analysis of secreted, membrane-processed, and pre-TSH subunits on SDS-polyacrylamide slab gels (35 cm). Lane 1, nonimmune serum added to reticulocyte lysates programmed with tumor mRNA (66 pg/ml), $[^{35}S]$Met, and stripped microsomal membranes (1.6 $A_{280}$ units/ml) after a 1-h incubation at 29°C. Lane 2, TSH ($\alpha$, downward arrow; $\beta$, upward arrow) secreted by cells in culture and immunoprecipitated with anti-$\alpha$ serum. Lane 4, membrane-processed proteins synthesized in lysate system as described in Lane 1. Lane 5, as in Lane 4 and immunoprecipitated with anti-$\beta$. Lane 6, as in Lane 5 except 10 pg of bTSH$\alpha$ were added before anti-$\alpha$. Lane 7, proteins synthesized in lysate as described in Lane 1, except without microsomal membranes present. Lane 8, as in Lane 7 and immunoprecipitated with anti-$\alpha$. Lane 9, proteins immunoprecipitated with anti-$\alpha$ serum from translation of tumor mRNA in wheat germ extract (Fig. 1, Lane 3). Lane 3, molecular weights as in Fig. 1 legend with the following additions: phosphorylase b (94,000), catalase (60,000), ovalbumin (43,000), lactate dehydrogenase subunit (36,000), and immunoglobulin light chain (24,000). Amounts of total and immunoprecipitated proteins loaded/sample well were as in Fig. 1 legend. Lanes 1 and 2 were from a different gel than were Lanes 4 to 9 and were aligned by protein standards on each gel.

### Table I

<table>
<thead>
<tr>
<th>Relative amounts of $[^{35}S]$Met-labeled proteins immunoprecipitated from wheat germ translations of thyrotropic tumor mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total $[^{35}S]$Met cpm/100-$\mu$l translation</strong></td>
</tr>
<tr>
<td>TCA-insoluble$^a$</td>
</tr>
<tr>
<td>Without added mRNA</td>
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<td>420,000</td>
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$^a$ 100-$\mu$l translations contained 150 pCi of $[^{35}S]$Met (1.28 $\mu$M). The results are an average of duplicate experiments which differed by less than 10%.

$^b$ Trichloroacetic acid-insoluble counts per min were determined from samples prepared for immunoprecipitation (14) before addition of antiserum.

$^c$ Optimal immunoprecipitations of pre-TSH$\alpha$ and pre-TSH$\beta$ were attained, respectively, with 10 $\mu$l of anti-$\alpha$ and 10 $\mu$l of anti-RCM TSH$\beta$ sera. Anti-TSH$\beta$ at various concentrations gave the same results; those shown are for 10 $\mu$l of anti-TSH$\beta$ serum/100-$\mu$l translation. Equivalent amounts of nonimmune serum were added as controls.

$^d$ Indicates immunoprecipitation without added, unlabeled authentic subunit. bTSH$\alpha + bTSH\beta$ indicates 10 pg of bTSH$\alpha$ or bTSH$\beta$ was added immediately before antiserum (competition experiments).
DISCUSSION

The data show that a TSHβ-immunoreactive protein (Mr = 15,500) is coded by about 5% of pituitary thyrotrophic tumor mRNA. This protein is nearly 2500 daltons larger than the protein core of authentic βTSHβ (31), suggesting it is a biosynthetic precursor, pre-TSHβ. Although authentic TSHβ is larger than α, the putative β precursor has an apparent molecular weight less than pre-TSHα (14). This may be due to different "signal peptide" lengths (see below) or anomalous mobilities on SDS gels (32), and a definitive explanation awaits complete chemical characterization of both precursors. Mg²⁺-dependent biosynthesis of a tumor protein postulated as pre-TSHβ (Mr = 15,000) has been reported (12); however, we did not observe an ion dependence of pre-TSHβ (Mr = 15,500) synthesis. It is difficult to compare the 17,000-dalton putative TSHβ precursor with the TSHβ-immunoreactive protein described here because the former was not immunologically or chemically defined (12).

The only other glycoprotein hormone β subunit identified and isolated by immunoprecipitation from cell-free translations is hCGβ (6). Its molecular weight (18,000) is about 2500 daltons larger than authentic hCGβ (33), and it has been suggested that it contains an NH₂-terminal signal peptide (6). Authentic hCGβ possesses 20 to 30 amino acids at its COOH terminus (33) that are not present in authentic β subunits of TSH, LH, or FSH of pituitary origin. Consequently, whether pre-TSHβ possesses an NH₂-terminal signal peptide as found with other presecretory proteins (34), including pre-α subunits (7, 14) and presumably pre-hCGβ (6), or whether it has an additional COOH-terminal sequence equivalent to that in authentic hCGβ remains to be determined.

Antiserum to isolated bTSHβ subunits recognize determinants of their homologous antigens in intact TSH (35). The data described here show that anti-α and -TSHβ sera recognize their homologous antigens in TSH secreted by tumor cells in culture (Fig. 3, Lane 2; Fig. 4A, Lane 1), and furthermore, these antisera recognize, respectively, α and β subunits processed co-translationally by homologous microsomal membranes (Fig. 3, Lane 5; Fig. 4A, Lane 5; Fig. 4B, Lane 3). These data support the suggestion that, although a conformational change may occur in one or both subunits accompanying subunit association (36), it is apparently not sufficient to mask antigenic determinants of the individual subunits when associated in intact TSH.

By contrast, anti-TSHβ serum fails to recognize pre-TSHβ. Rather, the antisera to denatured, reduced, and alkylated TSHβ recognizes pre-TSHβ (Fig. 1, Lanes 4 and 6) but not TSHβ processed by microsomal membranes or in combination with α in TSH (Fig. 4A, Lanes 2 and 5). It should be noted that the antigen for anti-RCM TSHβ production was prepared by disulfide reduction in the presence of 8 M urea and subsequent alkylation (24), a procedure that abolishes secondary and tertiary structure in the subunit (37). Presumably, the...
Cell-free Synthesis of TSHβ

...only antigenic determinants remaining reside in the linear sequence (24). Taken together, the data suggest that pre-TSHβ exists in a conformational difference from membrane-processed TSHβ or TSHβ combined with α in the intact hormone. The putative conformational transition may occur during transfer of the nascent β subunit across the rough endoplasmic reticulum. Studies on the refolding of TSHβ indicated that sufficient information may not reside in the primary structure of the authentic subunit (37,38), and similar results were obtained with hCGβ (37, 39). In this context it is striking that detection of pre-hCGβ synthesis was also achieved only with an antiserum to denatured hCGβ (6). It is not known whether a short sequence of amino acids exists at the NH₂ or COOH terminus in pre-TSHβ or -hCGβ, similar to those in proparathyroid hormone (40, 41) and other “proproteins” (42). If so, it will be of interest to examine whether the folding of nascent chains is influenced by such small propeptides or by extrinsic peptides, e.g. glutathione, that can form mixed disulfides with cysteine-containing proteins (43). Furthermore, whether there is an interaction between hydrophobic signal peptides and hydrophobic domains in precursors of proteins in general before or during processing by microsomal membranes remains to be determined.

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Evidence for conformational differences between precursor and processed forms of thyroid-stimulating hormone beta subunit.
L C Giudice and B D Weintraub


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