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Studies of Double-Labeled Mouse Thyrotropin and Free α-Subunits to Estimate Relative Fucose Content (42411)

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Abstract. The composition and structure of the complex oligosaccharides of thyrotropin (TSH) and free α-subunits are not well established, but are believed to be important determinants of the biological properties of these glycoproteins. We employed a simple double-label technique to learn the relative fucose content of mouse thyrotropin and free α-subunits. Thyrotropic tumor minces were incubated simultaneously with [^{35}S]methionine and [^{3}H]fucose. Thyrotropin and free α-subunits were labeled with both isotopes, and the ratio of ^{3}H/^{35}S was higher in free α-subunits than in thyrotropin; free α-subunits were approximately fivefold richer in fucose than was thyrotropin. The ^{3}H/^{35}S ratio was not substantially altered in TSH or free α-subunits secreted after a brief incubation with 10^{-7} *M* thyrotropin-releasing hormone. Species which incorporated [^{3}H]fucose were resistant to endoglycosidase H. Thus, mouse free α-subunits secreted by thyrotropic tumor are relatively rich in fucose. Double-isotope labeling using an amino acid and a sugar appears to be a useful technique for studies of the glycoprotein hormones.

Thyrotropin (TSH) is a glycoprotein that consists of two noncovalently linked subunits, α and β (1). Under certain conditions, thyrotrophs secrete excess α-subunits which are not combined with β-subunits, and are termed free α-subunits. It has been widely observed in many laboratories that free α-subunits migrate during gel electrophoresis with a slightly higher apparent molecular weight than α-subunits derived from the pituitary glycoprotein hormones (TSH, luteinizing hormone, and follicle-stimulating hormone), or from the placental hormone, chorionic gonadotropin. Considerable evidence suggests that the higher molecular weight of placental free α-subunits is a result of increased glycosylation (2), perhaps increased sialylation (3), or fucosylation (4, 5). Bovine pituitary free α-subunits contain *O*-linked carbohydrates not present in the α-subunits of the pituitary glycoprotein hormones (6), and may partially explain their increased molecular weight. Because incorporation of excess fucose might in part account for the high molecular weight of mouse free α-subunits, we employed a double-isotope labeling technique to seek differences in the molar content of fucose in mouse TSH as compared with that of free α-subunits. Studies of a glycoprotein dual-labeled with different isotopes in fucose and methionine have not, to our knowledge, been previously published.

Materials and Methods. Mouse pituitary thyrotropic tumors were incubated and transplanted as previously described (7, 8). In a typical experiment, a tumor from a single mouse (average tumor weight, 1 g) was minced to 1-mm^3 pieces, and preincubated for 60 min at 37°C in moist 5% CO_2, 95% O_2 atmosphere within a shaking water bath in sterile tubes containing serum-free, methionine-free Dulbecco's modified Eagle's medium having 20 mg/dl glucose. Labeling was initiated by addition of 625 μCi/ml of L-[^{35}S]methionine (Amersham/Searle, 800 to 1100 Ci/mmol) and 625 μCi/ml of L-[6-^{3}H]fucose (84 Ci/mmol; New England Nuclear). After 5 hr, the medium was collected, and tissues were washed with ice-cold phosphate-buffered saline, pH 7.4, before storing at −20°C. In one experiment, 10^{-7} *M* thyrotropin-releasing hormone (TRH) was added to minces 30 min before the isotopes, followed by a 5-hr incubation. Tissue minces were homogenized in a detergent-containing buffer, and TSH and free α-subunits were immunoprecipitated sequentially from lysates and media using specific antisera and *Staphylococcus A* (Pansorbin, Calbiochem, La Jolla, Calif.) as described previously (7, 8). Each specimen was incubated with anti-β serum first to obtain TSH + free β-subunits (only trace amounts of free β-subunits were present), followed by anti-α serum to obtain

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residual free α-subunits. Radioactivity was at background levels in precipitations using nonimmune serum. After subunits were eluted from the *Staphylococcus* pellets by boiling, each 100-μl specimen was divided equally and incubated with or without 5 mIU endoglycosidase H (Miles Scientific, Naperville, Ill.) (8). Subunits were analyzed by electrophoresis using SDS–polyacrylamide slab gels (7). Gels were sliced, and radioactivity was counted using a dual-isotope program.

Results. The results of a representative experiment are displayed in Figs. 1 and 2. In the lysates, TSH migrated with mol wt = 18–22K, and was well labeled with [^{35}S]methionine, but poorly labeled with [^{3}H]fucose (Fig. 1a). The free α-subunits derived from the very same lysate migrated with mol wt = 19–24K, and the species in the 22–24K region were better labeled with [^{3}H]fucose than with [^{35}S]-methionine (Fig. 1b). Treatment with endoglycosidase H, which cleaves high mannose but not complex-type oligosaccharides, caused some of the TSH subunits to shift to mol wt = 12K, the apparent weight of the apoprotein "core" of the α- and β-subunits (7, 9) (Fig. 1c). Likewise, endoglycosidase H caused a shift of some of the [^{35}S]methionine-labeled free α-subunits to mol wt = 12K (Fig. 1d). However, no [^{3}H]fucose-labeled free α-subunits shifted to mol wt = 12K, suggesting that fucose addition was correlated with the oligosaccharide resistance to the enzyme. Some [^{3}H]fucose-labeled free α-subunits were detected in the mol wt = 16–18K gel region, perhaps attributable to differential post-translational processing of the two *N*-linked oligosaccharides known to be present in free α-subunits, with one oligosaccharide being fucosylated while the other remained transiently a high mannose, endoglycosidase H-sensitive form.

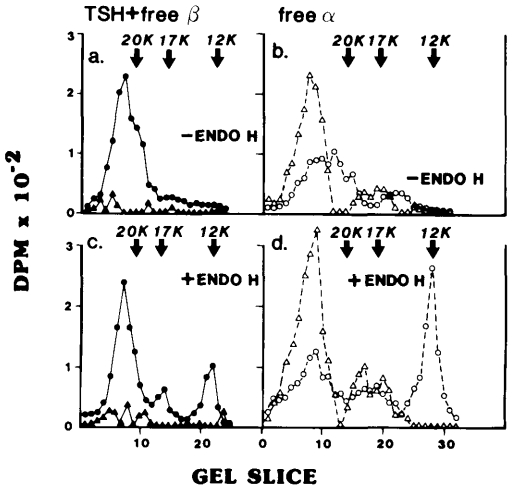

FIG. 1. Electrophoretic profiles of double-labeled TSH subunit precursors from tumor lysates. Mouse thyrotropic tumor tissue was incubated simultaneously with [^{35}S]-methionine and [^{3}H]fucose. TSH subunits were immunoprecipitated sequentially from tissue lysates, first using anti-β serum to obtain TSH heterodimers, and then anti-α serum to obtain residual free α-subunits. Species were then incubated in the absence (a, b) or presence (c, d) of endoglycosidase H, and analyzed by SDS–polyacrylamide gel electrophoresis. The arrows denote the positions of Coomassie blue-stained molecular weight markers that were added to each specimen immediately before electrophoresis. ●, ^{35}S, anti-β; ▲, ^{3}H, anti-β; ○, ^{35}S, anti-α; △, ^{3}H, anti-α.

TSH and free α-subunits that had been secreted into the medium are shown in Fig. 2. These data are taken from a different tissue incubation in which a more favorable ratio of high to low energy isotope was obtained. Free α-subunits displayed less heterogeneity in the medium than in the lysate; all [^{35}S]-methionine-labeled species comigrated with [^{3}H]fucose-labeled species (compare Fig. 2b with Fig. 1b). All secreted species were resistant to endoglycosidase H (not shown). Free α-subunits in the medium were apparently about fivefold richer in fucose than was secreted TSH. In a single experiment, TRH did not substantially alter the [^{3}H]fucose/[^{35}S]methionine ratio in secreted TSH or free α-subunits (not shown).

Discussion. Post-translational processing of glycoproteins having *N*-linked oligosaccharides generally involves the conversion of high mannose, endoglycosidase H-sensitive oligosaccharides to complex, endoglycosidase H-resistant oligosaccharides. The latter may contain fucose. Fucose has been detected in the glycoprotein hormones from several species, and it has been reported that TSH derived from mouse thyrotropic tumors contains one residue of fucose per molecule (10). Past reports of the carbohydrate compositions of the glycoprotein hormones have generally been based on biochemical analyses of carefully purified hormones. Such analyses are difficult, and are not applicable when only trace

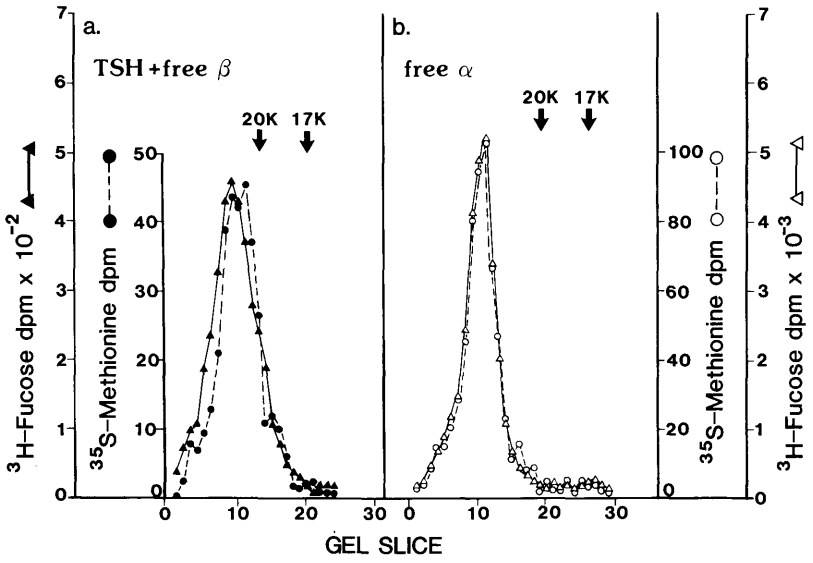

FIG. 2. Electrophoretic profiles of double-labeled TSH and free α-subunits immunoprecipitated from the medium. Mouse thyrotropic tumor tissue was incubated as described in the legend to Fig. 1. TSH and free α-subunits that had been secreted were sequentially immunoprecipitated from the medium, and were incubated in the absence (a, b) or presence (not shown) of endoglycosidase H before analysis by SDS–polyacrylamide gel electrophoresis. Note ordinate scales: free α-subunits were better labeled with [^{3}H]fucose than were TSH heterodimers. The arrows denote the positions of molecular weight markers.

amounts of product are available. Because radioactive fucose is not readily converted by tissues to other sugars during short incubations (11), we sought to gain insight into the relative fucose content of mouse TSH and free α-subunits by incubating thyrotropic tissue with [^{3}H]fucose and [^{35}S]methionine simultaneously. We interpreted fluctuations of the [^{3}H]/[^{35}S] ratio as reflecting variable fucose content, since the methionine content of these species is believed to be relatively fixed. We confirmed that substantial amounts of [^{3}H]-fucose were not converted to [^{3}H]mannose in these experiments because endoglycosidase H did not cleave the label from free α-subunits. Addition of [^{3}H]fucose to stored species not labeled with [^{35}S]methionine was believed not to occur in significant amounts, since these tumor cells were actively synthesizing and secreting TSH.

We found that TSH in lysates and media incorporated small amounts of [^{3}H]fucose, but that free α-subunits in lysates and media were relatively more rich in fucose. The increased fucose content of free α-subunits may in part explain their higher molecular weight as compared to α-subunits from mouse TSH heterodimers. Interestingly, workers using an entirely different technique making use of binding to lectins have found that free α-subunits from patients with pituitary tumors are highly fucosylated (12). On the basis of these and other data, we estimate that secreted free α-subunits were approximately fivefold richer in fucose than was TSH. Also, the [^{3}H]fucose/[^{35}S]methionine ratio in secreted TSH or free α-subunits was not altered by TRH in one experiment.

The α- and β-subunits derived from intact TSH were poorly resolved in these experiments, but in recent studies in our laboratory we found the relative fucose content in TSH β: α-subunits from the heterodimer to be approximately 2:1 (data not shown). This is in agreement with a recent report in which a double-label design was also employed (13). Of note, radioactive alanine and glucosamine have also been employed successfully in a double-label study of TSH (14).

The structures of the complex oligosaccharides of the glycoprotein hormones are not well established. The oligosaccharides influence the

rate of the metabolic clearance, as well as the intrinsic biological activity of the hormones. Of note, the fucose content of complex oligosaccharides correlates in many cases with more highly branched forms. The subcellular sites and mechanisms of biosynthesis, the hormonal regulation, and the physiological significance of the fucosylation of TSH and free α-subunits are active areas of research in our laboratory. A recent study has demonstrated that the subcellular site of the fucosylation of TSH may be modulated by the physiologic milieu of the thyrotroph (15). Use of a radioactive amino acid and a sugar to double-label hormone appears to be a convenient technique to assess carbohydrate composition, but future studies need to correlate findings with composition studies performed using biochemical techniques.

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