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FURTHER CHARACTERIZATION OF THE LOW AND HIGH AFFINITY BINDING COMPONENTS OF THE THYROTROPIN RECEPTOR*

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Following cross-linking with disuccinimdiyl suberate and analysis by SDS-PAGE and autoradiography, both the high- and low-affinity TSH binding components exhibited two similar ^{125}I-TSH-labeled bands, with Mr values of 80,000 and 68,000. IgG fractions from patients with Graves' disease inhibited ^{125}I-TSH binding to both components, while normal IgG had no effect. Although not entirely conclusive, these results suggest that the high- and low-affinity components share simlar subunit composition and antigenic determinants.

Equilibrium saturation analysis of the interaction of TSH with its receptor results in a nonlinear (concave upward) Scatchard plot (1-4). Kinetic and thermodynamic analyses of TSH binding have shown that this complex behavior is best explained by the existence of at least two binding sites; one exhibiting high affinity and low capacity, and the other low affinity and high capacity for TSH (4,5). Further studies have shown (6) that the high- (Ha) can be separated from the low- (La) affinity component by a number of methods, including gel filtration

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Abbreviations: DSS, disuccinimidyl suberate; PMSF, phenylmethylsulfonyl fluoride; STI; soybean trypsin inhibitor; DFP; diisopropyl fluorophosphate; PPM, partially-purified membranes; PM, purified membranes; SM, solubilized membranes; SDS-PAGE, sodium dodecyl sulfate - polacylarylamide gel electrophoresis; DTT, dithiothreitol; Ha and La, high- and low-affinity binding components, respectively.

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chromatography on Sepharose 6B, lectin affinity chromatography, and discontinuous sucrose density gradient centrifugation. Although Ha appears to be the receptor responsible for activation of adenylate cyclase (7-9), neither the chemical identity nor the physiological role of La has been established.

The present investigation was initiated to identify the subunit structure of the high- and low-affinity components of TSH receptor and to elucidate their mode of interaction with anti-TSH receptor antibodies of Graves' sera. Our results show that (a) binding of ^{125}I-TSH to Ha and La is inhibited by Graves' IgG and (b) although differing in several properties, including size (1,4-6), the two components each contain two similar protein subunits with Mr values of 80,000 and 68,000.

MATERIALS AND METHODS

Purified bovine TSH (40 International Units/ mg), a gift from Dr. John Pierce (UCLA), was iodinated with ^{125}I (Amersham IM-30) to a specific radioactivity of 50-90 μCi/μg by a modification of the lactoperoxidase method, as previously described (1). Disuccinimidyl suberate (DSS) was purchased from the Pierce Chemical Co. (Rockford, IL). Porcine thyroids were obtained from the Pel-Freeze Co. (Rogers, AR). DEAE Affi-Gel Blue was purchased from Bio-Rad (Richmond, CA). All other chemicals were of the highest quality commercially available.

Preparation of Purified and Solubilized Membranes

Partially purified membranes (PPM) were prepared as previously described (1), except for the presence of the protease inhibitors, 2.0 mM diisopropyl fluorophosphate (DFP), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg/ml soybean trypsin inhibitor (STI)), and stored at -90°C. PPM were subsequently purified on discontinuous sucrose density gradients, as previously described (10). The purified membrane pellets (PM) were resuspended in 10 mM sodium phosphate buffer, pH 7.4, containing 0.5 mM DFP, 1 mM PMSF, and 0.1 mg/ml STI (Buffer A) at an approximate concentration of 1 mg/ml. Protein concentrations were determined by the Lowry method of (11). Membranes were solubilized in 1% Triton X-100 in Buffer A (6). Solubilized membranes (SM) were stored at -90°C at an approximate protein concentration of 1-2 mg/ml. Protein concentrations were determined by the Markwell modification of the Lowry method (12).

Binding and cross-linking of ^{125}I-TSH to purified membranes

Binding of ^{125}I-TSH to thyroid PM was carried out in Buffer A in the presence or absence of excess unlabeled TSH, as described previously (1). Radioiodinated TSH was covalently linked to PM by a modification of the disuccinimidyl suberate method of Pilch and Czech (13), as described previously (14). Labeled membranes were then solubilized in 1% Triton X-100 in Buffer A, and the 100,000 xg supernatant containing solubilized membranes was fractionated by centrifugation on discontinuous sucrose gradients as described previously (6). The gradients were fractionated from the bottoms of the tubes and bound ^{125}I-TSH was assayed by precipitation with polyethylene glycol and filtration (6,15). Aliquots from the sucrose gradient peaks of radioactivity corresponding to the low and high affinity

binding components were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (16). Statistical analysis was made using Student's t test or analysis of variance. Statistical significance was established at $P < 0.05$.

RESULTS AND DISCUSSION

Cross-linking of ^{125}I-TSH to purified thyroid membranes with DSS followed by analysis on discontinuous sucrose density gradients resulted in the appearance of two peaks of specifically bound ^{125}I-TSH (Fig.1). The lighter peak (peak I) represents Ha while the heavier peak (Peak II) is La, as previously demonstrated by chromatography on Concanavalin A-Sepharose columns followed by Scatchard analysis (6). However, since the labeling of the La was relatively low and could not be visualized by autoradiography, the procedure was therefore modified to maximize the labeling of this component. To minimize the dissociation of labeled TSH

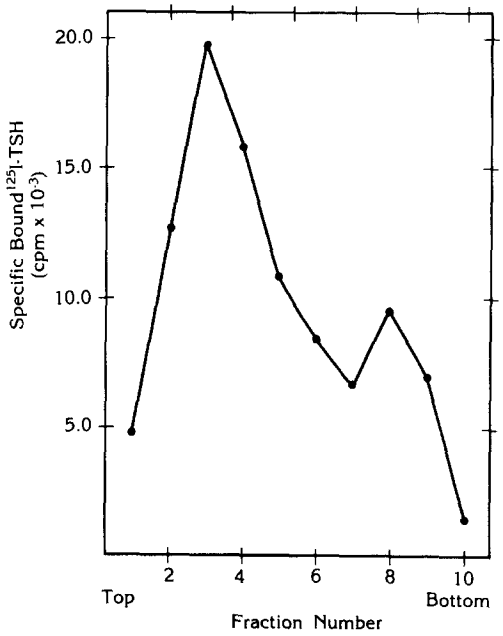
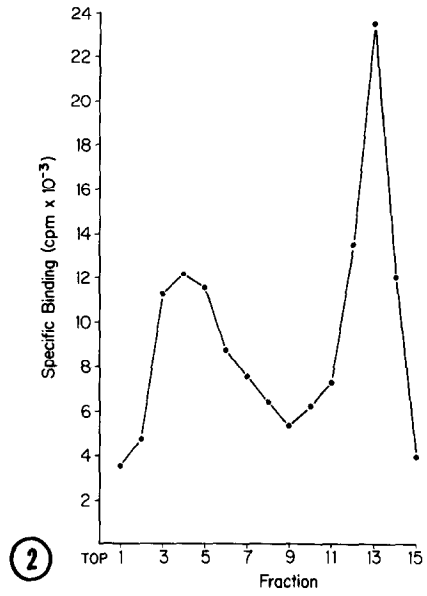


Figure 1: Discontinuous sucrose density gradient ultracentrifugation of SM covalently cross-linked to ^{125}I-TSH. Thyroid PM (1 mg) were incubated with ^{125}I-TSH (0.6 nM) in the presence or absence of 1.25 IU of native TSH for 1 h at room temperature. Following removal of unbound TSH by washing and centrifugation, ^{125}I-TSH was covalently linked to the membrane by incubation with 0.3 mM DSS as described in the text. The labeled membranes were washed and then solubilized with 1% Triton X-100. The labeled SM were layered on top of discontinuous gradients of 5,10,20 and 50 percent (v/v) sucrose and were centrifuged at 22,000 x g for 2 h at 4°C. The gradients were fractionated from the bottom of the tube, and each aliquot was assayed for bound ^{125}I-TSH by PEG precipitation and filtration. Bound radioactivity was corrected for nonspecific and filter binding. Results were expressed as the average of duplicate determinations.



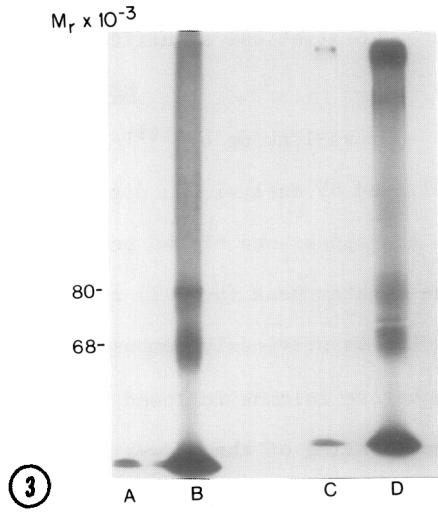


Figure 2: Discontinuous sucrose density gradient ultracentrifugation of thyroid PM following labeling with radioiodinated TSH. Thyroid PM (1 mg) were labeled with 2.4 nM ^{125}I-TSH and cross-linked as described in Fig. 1. However, the membranes were not washed to remove unbound TSH prior to cross-linking. Following solubilization with Triton X-100, the membranes were analyzed as described in Fig. 1.

Figure 3: Electrophoretic mobility of the ^{125}I-TSH labeled high- and low-affinity binding components. Aliquots of the binding peaks corresponding to the covalently labeled high- and low-affinity binding components in Fig. 2 were boiled in 2% SDS containing 50 mM DTT. The labeled membranes were then subjected to SDS-PAGE on 7.5% acrylamide gels. Following electrophoresis, the gels were dried and analyzed by autoradiography. High- and low-affinity binding peaks cross-linked to ^{125}I-TSH in the presence (lane A and C) or absence (lanes B and D) of excess unlabeled TSH, respectively.

from La, unbound TSH was not removed by washing of the labeled membranes prior to cross-linking with DSS. As can be seen in Fig. 2, this procedure increased the specific labeling of La in comparison to that of Ha. Electrophoresis and autoradiography of aliquots of the peaks corresponding to Ha and La resulted in the appearance of two similarly labeled bands with Mr values of 80,000 and 68,000 (Fig. 3, Lanes B and D). These two bands have previously been identified to represent the main binding subunits of the TSH receptor (14). The band near the bottom of the gels has also been identified as unbound TSH (14). The bands on the top of the gels could not be resolved in 5% acrylamide and therefore were assumed to be protein aggregates. The labeling of the 80,000 and 68,000 bands was specific, for when the membranes were incubated in the presence of excess native

hormone, the bands were not observed (Fig. 3, Lanes A and C). Occasionally a third labeled band, with M_r of 200,000, was also seen on gels of the low affinity peak. This band was observed in the presence of DTT (50 mM), suggesting that it was not composed of disulfide-linked subunits. Similar peptide has also been occasionally observed in SDS extracts of intact thyroid membranes following labeling with ^{125}I-TSH and cross-linking with DSS (14). The identity of this band is still unknown; however it may either represent some molecules of the TSH holoreceptor whose subunits were crosslinked with DSS or it may represent a component of the receptor that is not efficiently cross-linked with DSS. Alternatively, the 200,000 band may represent some of the TSH holoreceptor that was not dissociated upon boiling in SDS (16,18). Similar observation has previously been reported by Islam *et al.* (10) who showed that the TSH holoreceptor (M_r = 197,000) is a noncovalently-linked oligomer, some of which is not denatured by boiling in SDS.

It is well established that the sera of patients suffering from Graves' disease possess several autoimmune antibodies, some of which appear to be directed against the TSH receptor (for review see ref. 20). Some of these antibodies have previously been shown to inhibit the binding of ^{125}I-TSH to thyroid membranes (14,20) as well as to the 80,000 and 68,000 Mr bands (14). This ability to inhibit binding was utilized to further analyze the physicochemical properties of La and Ha. Protease-free IgG fractions of normal sera and sera of patients with Graves' disease were prepared by chromatography on DEAE Affi-Gel Blue, and tested for their ability to inhibit ^{125}I-TSH bindings to thyroid membranes, as described previously (14). In agreement with previous findings (20,14), the IgG fractions from some Graves' disease patients inhibited the binding of ^{125}I-TSH in a dose-dependent fashion ($P < 0.001$), while normal IgG had little effect (data not shown). These IgG fractions were then examined for their ability to block the binding of ^{125}I-TSH to the solubilized high and low affinity binding components. Solubilized

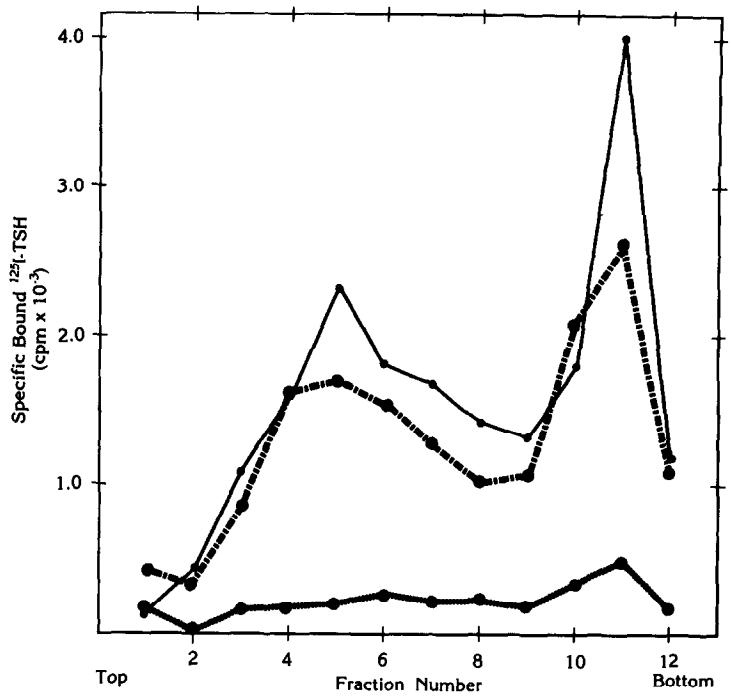


Figure 4: Effects of IgG on the labeling of the high and low affinity TSH binding components. Thyroid SM (0.5 mg) were preincubated with buffer (●——●), normal (●-·-·-·-·-●) or Graves' (●▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪●) IgG (1.5 mg) followed by incubation for an additional hour with ^{125}I-TSH (0.6 nm) in the presence or absence of excess unlabeled hormone,(1.25 IU). The labeled membranes were then analyzed on discontinuous sucrose gradients as described in Fig. 1.

thyroid membranes were preincubated with buffer (control),normal or Graves' IgG for 1 h at room temperature. The membranes were then labeled with ^{125}I-TSH and analyzed on discontinuous sucrose density gradients. As can be seen in Fig. 4, preincubation with IgG from normal sera caused a slight decrease in the labeling of both peaks ($P > 0.05$). However, IgG from Graves' disease patients resulted in marked inhibition of the labeling of both peaks ($P < 0.001$). Although not entirely conclusive. these data suggest that La and Ha are structurally related and possibly interact with Graves' IgG through similar antigenic determinants. This latter conclusion is supported by the suggestion that Graves' anti-TSH receptor antibodies are highly homogeneous arising against a single antigenic determinant on the receptor (20).

Previous evidence indicates (7-9) that Ha is the receptor required for adenylate cyclase activation by TSH, whereas La does not appear to

play any significant role in this interaction. Since La is greater in size than Ha (6), it is possible that La represents an aggregate of the receptor induced by hormone action, a phenomenon similar to that previously demonstrated for insulin (21,22). Alternatively, since Ha binds to Concanavalin A and La does not, La may represent a metabolic precursor of Ha (23). It is conceivable that La contains co-translationally added core oligosaccharide units that are subsequently processed by the Golgi apparatus to mannose containing glycoproteins that bind concanavalin A. However, it is unlikely that the low affinity component is formed as an artifact of the membrane preparations, since it has recently been demonstrated on intact FRTL-5 rat thyroid cells (Gennick, Thomas and Nayfeh, unpublished results).

In conclusion, the present results confirm and extend our previous findings (6) that TSH-receptor complexes exist in two distinct forms that differ in several physicochemical properties, including size, binding affinity and carbohydrate composition. The participation of multiple forms of receptors appears not to be unique for the interaction of TSH with thyroid plasma membranes, but includes the interaction of such hormones as catecholamines, insulin, dopamine, angiotensin, and glucagon with their target cells (see ref. 24 for additional discussion). Using wheat germ lectin-Sepharose columns, Mason and Tager (24) have recently reported the separation of high- and low-affinity binding components for the hepatic glucagon receptor that differ in their interaction with wheat germ lectin and guanine nucleotides. It will be of interest to determine the degree of involvement of the low-affinity component in the responsiveness of target cells to hormone action, using monoclonal antibodies raised against this component as well as tumors lacking low affinity binding (7,8).

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