Structure of the Thyrotropin Receptor and Thyroid Adenylate Cyclase System As Determined by Target Analysis†

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ABSTRACT: Bovine thyroid plasma membranes were irradiated with high-energy electrons. Analysis of the target size of the thyrotropin (TSH) receptor revealed a complex pattern composed of a TSH binding component of 71 000 daltons and a large component (several hundred thousand daltons) that masked some of the binding. Both components were also observed when binding was assayed in the presence of 50 mM NaCl. Membranes preincubated with Mg^{2+} and 10 μM guanosine 5′-(β,γ-imidotriphosphate) [Gpp(NH)p], a persistent activator of adenylate cyclase, also showed the presence of these same components. Although the receptor for TSH has been reported to have some similarities to the receptor for cholera toxin, target analysis of [^{125}I]iodocholera toxin binding was consistent with a single small component about the size of a ganglioside. Measurement of the target size of ground-state, i.e., not preactivated, adenylate cyclase was also carried out. The basal (Mn^{2+}) adenylate cyclase yielded a M_r of 85 000, the smallest unit capable of producing cAMP. The Gpp(NH)p-responsive adenylate cyclase has a M_r of 150 000, which may reflect the contribution of the guanine nucleotide regulatory component to the mass of the active enzyme. A similar size was previously measured for the Gpp(NH)p-preactivated, detergent-solubilized thyroid enzyme [Asbury, R. F., Cook, G. H., & Wolff, J. (1978) *J. Biol. Chem. 253*, 5286–5292]. Radiation inactivation of the NaF-responsive enzyme indicated two or more components to this activity, the smaller of which (140 000 daltons) was similar in size to the ground-state Gpp(NH)p-responsive enzyme and the larger of which was greater than 10^6 daltons. TSH stimulation of adenylate cyclase was also consistent with the presence of two or more components, the smallest being 240 000 daltons and the largest being greater than 10^6 daltons. These structures may correspond to a contribution of the hormone receptor (71 000 daltons) and the combination of nucleotide regulatory component and catalytic unit (150 000 daltons). Thus, the structure of the thyroid adenylate cyclase appears to be as complex as the adenylate cyclase from rat liver and more complex than turkey erythrocyte adenylate cyclase.

The thyrotropin- (TSH)[1] responsive adenylate cyclase consists of the hormone receptor, a stimulatory inosine or guanine nucleotide regulatory (i.e., N) component, and a catalytic (or C) component. Many details of the structure are as yet unclear. Pekonen & Weintraub (1979) and Powell-Jones et al. (1980) have introduced several improvements in the conditions used for assessing TSH binding. Under these conditions, two classes of binding sites for TSH are apparent (Pekonen & Weintraub, 1979; Powell-Jones et al., 1980). The capacity of the lower affinity site is diminished at high ionic strength (Pekonen & Weintraub, 1979) while the higher affinity site appears necessary for activation of adenylate cyclase (Saltiel et al., 1980). Stimulation of the enzyme by TSH requires an inosine or guanine nucleotide such as GTP or the poorly hydrolyzed analogue Gpp(NH)p (Saltiel et al., 1981; Totsuka et al., 1982), but the enzyme may also be activated by NaF or Gpp(NH)p without hormone. Some reports have suggested similarities between the receptors for TSH and cholera toxin (Kohn, 1978) or that the receptor for the TSH may consist of (Mullin et al., 1978), or contain (Kohn et al., 1981), a ganglioside.

Previous analyses of the structure of thyroid adenylate cyclase have depended on stabilization of the enzyme by preincubation with activators and have not measured the size of the ground-state (nonactivated) enzyme (Asbury et al., 1978; Goldhammer et al., 1980). Preactivation of the thyroid adenylate cyclase with Gpp(NH)p results in formation of a discrete 159 000-dalton complex (NC), which is stable to solubilization in Triton N-101 (Asbury et al., 1978). A smaller molecular weight was found for the enzyme preactivated with NaF after solubilization (119 000) (Goldhammer et al., 1980). This suggested a difference in the composition of the enzyme complexes activated by the two agents, although both agents probably stimulate the enzyme through the N component (Spiegel & Downs, 1981; Ross & Gilman, 1980; Rodbell, 1980; Nielsen et al., 1980).

In this paper we report the molecular weights of the structures responsible for TSH binding and stimulation of adenylate cyclase activity determined by measuring the target size of these functions. The technique involves inactivation of a portion of the receptors or enzymes by graded doses of ionizing radiation. The molecular weight of the functional unit responsible for the activity may be calculated (Kempner & Schlegel, 1979) by measuring the radiation dose and the activity or specific binding at each dose.

Target-size analysis assumes that an ionization occurring anywhere in a functional unit results in complete loss of activity (Kempner & Schlegel, 1979). The physical and chemical state of the target molecule may modify its radiation sensitivity and, hence, its measured size (Kempner & Haigler, 1982; Parkinson & Callingham, 1982). Thus, this measurement alone cannot

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[1] Abbreviations: TSH, thyrotropin or thyroid-stimulating hormone; N, guanine or inosine nucleotide binding component responsible for the stimulation of adenylate cyclase and for the transduction of the hormonal signal (variously called N_s, G, G/F, G-unit, or G-protein); Gpp(NH)p, guanosine 5′-(β,γ-imidotriphosphate); SDS, sodium dodecyl sulfate; C, catalytic component of adenylate cyclase; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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identify the number or mass of any substructure within the functional unit, and interpretation of the measurements must depend on what is known of the biochemistry of the system (Schlegel et al., 1979). Postirradiation rearrangements and exchanges of subunits can also affect the measured sizes (Swillens & Dumont, 1981; Simons et al., 1982). In this study, the results suggest that the receptor is unexpectedly complex, composed of at least two macromolecular components: a binding component and component(s) that mask(s) the binding. The target sizes of the Gpp(NH)p- and NaF-responsive adenylate cyclase appear similar to the hydrodynamic size of the Gpp(NH)p-preactivated enzyme determined after solubilization with detergent (Asbury et al., 1978) but larger than the size of the solubilized NaF-preactivated enzyme (Goldhammer et al., 1980).

Experimental Procedures

Materials. $Na^{125}I$ (13–17 mCi/μg) was obtained as a carrier-free solution from Amersham (Elk Grove, IL). TSH (21 units/mg, NIADDK b-9) was generously provided by the Hormone Distribution Program of NIADDK. Dr. John Pierce of the University of California (Los Angeles, CA) graciously donated the bovine TSH (30 units/mg) used for iodination. Cholera enterotoxin was purchased from Schwarz/Mann (Orangeburg, NJ) or Sigma (St. Louis, MD); the two preparations were equivalent in displacing bound[^{125}I]iodocholera toxin. Dr. Lutz Birnbaumer (Molecular Endocrinology Core Laboratory of the Diabetes and Endocrinology Research Center, Baylor College of Medicine) kindly supplied the [α-^{32}P]ATP (>50 Ci/mmol) used for adenylate cyclase determinations. ATP (A-2383) was obtained from Sigma and was prepared by phosphorylation of adenosine and, therefore, has a low contamination of GTP. Thin-wall glass ampules (2-mL capacity) were manufactured by Kimble (Toldeo, OH). All other chemicals were reagent grade or the best available.

Plasma Membrane Preparation. Bovine thyroid plasma membranes were prepared by the sucrose gradient method of Yamashita & Field (1970). The membranes (at a concentration of 6–10 mg/mL) were frozen in dry ice and stored at −60 °C. Although the preparations were irradiated and assayed within a few days of preparation, the membranes retain TSH-responsive adenylate cyclase for over 6 months when stored in this manner.

Sample Irradiation. Prior to irradiation, the membranes were diluted to 2.8–3.0 mg/mL with 20 mM Tris–HCl, pH 7.4, and aliquots of up to 200 μl distributed into glass ampules, quick frozen, and sealed, as described previously (Schlegel et al., 1979). The membranes were transported in dry ice and subjected to graded doses of 10-MeV electrons at the Armed Forces Radiobiology Research Institute, Bethesda, MD, while the temperature was monitored and maintained at −135 ± 2 °C by N_2 gas flowing from a liquid N_2 reservoir. Controls were treated the same but received no radiation. The dose of radiation was routinely measured with thermoluminescent dosimeters and, on occasion, with radiation-sensitive dye films. Prior to assay, the ampules were opened, gassed with fresh air, thawed by being warmed with the hand, and rapidly diluted with a solution of 15 μM bovine serum albumin–20 mM Tris-HCl, pH 7.4, so that the membrane protein concentration was 0.8 mg/mL for samples to be used for adenylate cyclase assays. Aliquots were diluted to 0.02–0.05 mg/mL for the determination of TSH binding. Samples used for cholera toxin binding were diluted to 0.002 mg/mL. Appropriate aliquots were used for the assays as described subsequently.

Preparation of [^{125}I]Iodo-TSH. Purified TSH (30 units/mg) was iodinated for 30 s with $Na^{125}I$ by the lactoperoxidase method (Kotani et al., 1975). The product was immediately purified on Sephadex G-25, followed by binding to membranes, by dissociation by 2 M NaCl, and, lastly, by chromatography on Sephadex G-100 (Rees Smith et al., 1977). The specific activity was 60–100 mCi/mg. The purified [^{125}I]iodo-TSH was used within 2 weeks.

Assay of Binding of TSH. Two methods were used to assay the binding of TSH to bovine thyroid plasma membranes. Method 1 was as follows. Purified membranes (0.002–0.005 mg) were incubated for 30 min at 30 °C with about 10000 cpm (20–35 microunits/mL) of [^{125}I]iodo-TSH in a total volume of 250 μL containing 20 mM Tris-HCl, pH 7.4, and 15 μM bovine serum albumin. Method 2 was as follows. Purified membranes (about 0.015 mg) were incubated as above but in a buffer containing 50 mM NaCl, 15 μM bovine serum albumin, and 10 mM Tris-HCl, pH 7.4. For both methods, after the incubation the membranes were pelleted at 10000*g* for 5 min at 24 °C in a Beckman microfuge. The supernate was aspirated, 300 μL of cold solution (10% w/v sucrose, 20 mM Tris-HCl, pH 7.4) was layered over the pellet, and the samples were again centrifuged. After the supernate was removed, the samples were washed again with sucrose solution. The radioactivity remaining in the pellet and the tip of the plastic tube was counted in a gamma counter. Blanks (no membrane) were routinely less than 1.5% of the total radioactivity in the assay. Total binding was 10–15% of the total counts. The saturable [^{125}I]iodo-TSH binding ("specific binding") was typically greater than 80% of total binding when assessed as the difference in binding in the absence and presence of excess cold hormone (400 milliunits of TSH/mL). Specific and nonspecific binding reached an equilibrium by 30 min and was linear over the range of protein concentrations used.

Preincubation with Gpp(NH)p and Mg^{2+}. Where indicated, thyroid plasma membranes (0.2 mg/mL) were incubated for 5 min at 30 °C in 2×10^{-5} M Gpp(NH)p, 3.5 mM $MgCl_2$, 0.5 mM EDTA, and 20 mM Tris-HCl, pH 7.4, prior to freezing aliquots for irradiation. Upon being thawed, the binding of TSH to these samples was assessed. This treatment results in persistent activation of the adenylate cyclase activity (Asbury et al., 1978).

Preparation of [^{125}I]Iodocholera Toxin and Assay of Binding. Cholera enterotoxin was purified on Sephadex G-50 and iodinated as described by Cuatrecasas (1973). The specific activity was >400 Ci/mmol. By use of SDS gel electrophoresis (Laemmli, 1970), followed by autoradiography, two bands corresponding to the two polypeptide constituents of cholera toxin were labeled. Binding of [^{125}I]iodocholera toxin to purified thyroid plasma membranes was measured by incubation for 2 h at 22 °C in a final volume of 200 μL containing 140 fmol of [^{125}I]iodocholera toxin (about 120000 cpm), 90–110 ng of plasma membrane protein, 10 mM Tris-HCl, and 15 μM bovine serum albumin in Krebs–Ringer bicarbonate buffer (pH 7.3), except as noted in the figure legends. Identical assays containing 5 μg of cholera toxin (purified by chromatography on Sephadex G-50) were used to determine nonspecific binding. Bound and free cholera toxins were separated as described above for TSH. With this method, the blank was routinely about 1%, the total binding about 30%, and the nonspecific binding about 1–2% of the total radioactive cholera toxin in the assay. A linear relationship between cholera toxin binding and thyroid plasma membrane protein up to 180 ng/200 μL was observed. The amount of cholera toxin bound was 0.012 g of cholera toxin/g of membrane protein in several membrane preparations, and the labeled cholera toxin was

Table I: Functional-Unit Molecular Weights Derived from Target Analysis Curves of Data Grouped by Membrane Preparation

function	components I[a] M_r[b]	components I[a] dose range (megarads)	components I[a] possible constituents[c]	components II[a] M_r[d]	components II[a] dose range (megarads)
specific TSH binding					
at low ionic strength	71 000 ± 6000 (6)	18–72	R_{TSH}	>250 000 (5)	0–3
at high ionic strength	68 000 ± 6000 (3)	18–60	R_{TSH}	>260 000 (3)	0–3
specific cholera toxin binding	<5000 (3)	0–72		NP[e]	
adenylate cyclase					
basal, Mn^{2+}	85 000 ± 7000 (4)	12–72	C	150 000 (4)	0–3
NaF	140 000 ± 19 000 (6)	12–72	N, C	>10^6 (6)[f]	0–3
Gpp(NH)p	150 000 ± 18 000 (6)	0–72	N, C	NP[e]	
TSH responsive	240 000 ± 28 000 (5)	9–30	R_{TSH}, N, C	>10^6 (5)[f]	0–3

[a] Components I are calculated from data at high radiation doses, as described. For curves that are not simple exponentials, the sizes of the components II are listed. The component II for the binding assay bears no *necessary* relationship to any of the other components II. [b] The mean ± SE of the molecular weights of the functional units with the number of experiments in parentheses. [c] Possible constituents of components I are binding component of the receptor for TSH (R_{TSH}), catalytic unit of adenylate cyclase (C), and guanine or inosine nucleotide binding component responsible for the stimulation of adenylate cyclase (N). [d] The average molecular weight calculated from data at the low radiation doses described with the number of experiments in parentheses. [e] NP means not present; i.e., Figures 4 and 7 indicate the presence of only one component with each curve. [f] The data are corrected for the contribution of the smaller component by extrapolation of the regression line (obtained at high doses) to the y intercept and subtraction of the measured activity at each dose.

displaced by unmodified cholera toxin in a dose-dependent manner.

Adenylate Cyclase Assay. Adenylate cyclase activity was measured as the formation of [^{32}P]cAMP from [α-^{32}P]ATP during a 5- or 10-min incubation at 30 °C. The incubation medium in a total volume of 0.11 mL contained 0.04–0.048 mg of purified membrane protein, 1 mM ATP [containing (3–6) × 10^6 cpm of [α-^{32}P] ATP], 1.5 mM cAMP, 0.5 mM EDTA, 15 μM bovine serum albumin, 30 mM Tris-HCl, pH 7.4, and a nucleotide-regenerating system consisting of 0.9 mM phosphoenolpyruvate and 0.1 mg/mL pyruvate kinase (120 units/mL). Basal activity was measured in the presence of 2.5 mM $MgCl_2$: stimulated activities were measured in the presence of 3.5 mM $MgCl_2$. Activators, when used, were added to final concentrations of 90 milliunits/mL TSH, 10 μM Gpp(NH)p, or 10 mM NaF. The reactions were stopped by addition of 0.1 mL of a solution containing 4 mM ATP, 4 mM ADP, 4 mM AMP, and 4 mM cAMP, and then the tubes were heated at 90 °C for 3 min. An internal standard ([^{3}H]cAMP, 10 000 cpm) and 0.5 mL of water were added. After centrifugation of the tubes for 10 min at 2500 rpm at 4 °C in a Sorvall RC2 centrifuge, the cAMP in the supernate was isolated by a modification (Bockaert et al., 1976) of the chromatography procedure of Salomon et al. (1974) and counted in a Packard Tri-Carb scintillation counter. Thyroid adenylate cyclase assayed in this manner gave a linear production of cAMP for at least 15 min. The activity was proportional to membrane protein up to 0.06 mg/assay.

Calculation of Data. Hormone bound to the membranes (B) was divided by the free (F) hormone present, according to Rodbard (1973). Data of binding or activity after irradiation were analyzed by regression of ungrouped data (Schlegel et al., 1979) and of data grouped by membrane preparation (Nielsen et al., 1981). Data were tested for biphasic or monophasic character by computing the regression lines for the low-dose, high-dose, and combined data and comparing the regressions by the F test (Neter & Wasserman, 1974). It is important to note that our evaluation of the data assumes that little rearrangement of components occurs either in the frozen (−135 °C) state or prior to assay, in accord with the observations of Schlegel et al. (1979). Thyroid adenylate cyclase, unlike turkey erythrocyte adenylate cyclase (Nielsen et al., 1981), has appreciable activity in the presence of Gpp(NH)p. Thus in assessing the influence of TSH in the presence of Gpp(NH)p, the activity with Gpp(NH)p alone was subtracted from the activity with TSH plus Gpp(NH)p. Data for binding or activity were obtained up to the highest doses that gave values above background. Thus, assays with high activity relative to background were inactivated to a small fraction of initial activity. Uncertainty in the data is expressed as the standard error of the mean (SE), except as noted.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Results

TSH Binding Component. The effect of high-energy irradiation of thyroid plasma membranes on specific TSH binding measured at low ionic strength (method 1, see Experimental Procedures) produces a complex curve (Figure 1). At high doses of radiation, the binding is lost as a simple exponential function of the dose, consistent with a single target, here called component I. From several membrane preparations, the average M_r (using the grouped analysis) of this binding component I was 71 000 ± 6000 (Table I). The same data not grouped by preparation gave a value of 67 000–69 000 daltons. At low doses of radiation (less than 3 megarads), a significant increase in TSH binding was noted (Figure 1), but this increase was never more than 25% of the nonirradiated controls. Of several possible explanations for the phenomenon, the simplest is that the enhancement of binding at low doses of radiation results from the destruction of large component(s), which mask(s) the binding of TSH to the binding component. To estimate the size(s) of this (these) putative larger component(s) [here called component(s) II], the observed binding at low radiation doses was subtracted from the extrapolated line predicted for the smaller component I (Figure 1). Because the result is a small difference between two large numbers, there is considerable error in analysis of larger component(s). Therefore, we analyze only for the simplest case of a single-size component II. In order to obtain a minimum size estimate, we calculated the regression line for such data from samples receiving 0–3 megarads, leading to an estimated target of >250 000 daltons for the masker (Table I).

When TSH binding was measured in the presence of 50 mM NaCl (Figure 2; method 2, see Experimental Procedures), total specific binding was reduced (Pekonen & Weintraub, 1979), and the inactivation curve still had both of the distinctive

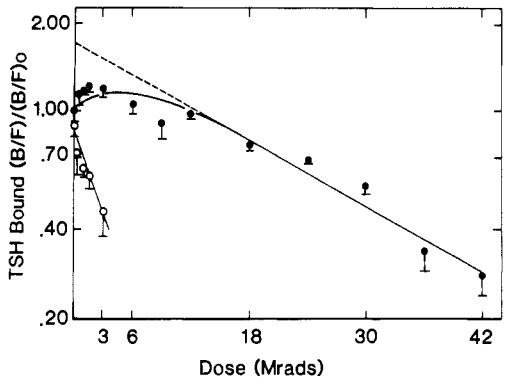

FIGURE 1: Radiation inactivation of the TSH receptor. Samples of bovine thyroid plasma membranes were irradiated in triplicate at each dose indicated, then thawed, and diluted, and each sample was assayed in duplicate for TSH binding by method 1 (low ionic strength) as described under Experimental Procedures (●). The specific binding is normalized to the nonirradiated controls, which bound 96 microunits of TSH/mg of membrane protein. Samples irradiated at doses of 0.5, 1.0, and 1.5 megarads showed a significant ($p < 0.05$) increase in binding compared to nonirradiated controls. The difference between the extrapolated line and the data is shown by the open circles (○). The data shown as the mean ± SE are representative of five of six preparations irradiated on separate occasions. (One preparation showed no increase in binding at low doses.) The data presented in Figures 1, 3, and 7 are from the same membrane preparation.

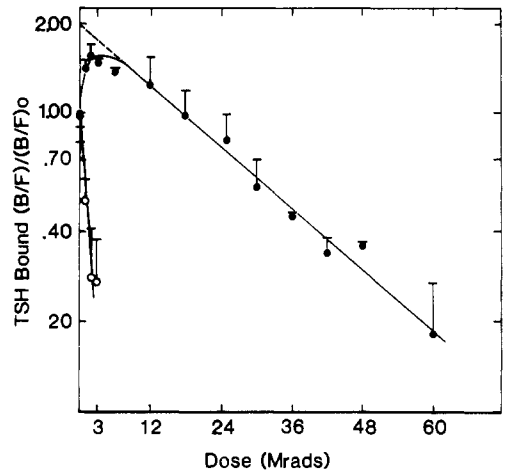

FIGURE 2: Radiation inactivation of the TSH receptor. After irradiation of duplicate bovine thyroid plasma membranes at the doses indicated, the binding of TSH was assessed in triplicate by method 2, (high ionic strength) as described under Experimental Procedures. The specific binding is normalized to the nonirradiated controls (which bound 6 microunits of TSH/mg of membrane protein) and plotted as the mean and range/2 (●). The difference between the extrapolated line and the data is shown by open circles (○). The data are representative of three preparations.

features found at lower ionic strength. That is, low doses (about 3 megarads) of radiation enhanced the binding relative to unirradiated controls; the increase in binding was always greater when assayed by method 2 than by method 1. The slope at high doses was similar to that in Figure 1, leading to a M_r 68 000 for the TSH binding component (Table I).

The parameters that should affect this increase in binding are the slope at low doses, the slope at high doses, and the relative abundance of each component (for convenience called binding component and masker). The slope associated with the small component is very similar in different preparations, suggesting that the change in activation results from either a change (with ionic strength) of the size of the masker or a change in the masker to binding component ratio. Let m represent the size of the masker and r the size of the binding component. Here we assume that the masked receptor has no binding activity. Since the initial preparation receptor activity *is* observed, some "unmasked" binding components must be present. If all the maskers could be selectively removed, there would be an increase in observed binding up to that of all the available binding components. This would correspond to the intercept (dose = $D = 0$) of the extrapolated lines in Figures 1 and 2. Thus

$$A = A_T e^{-rD} - Be^{-mD} \quad (1)$$

where A is the observed TSH binding, A_T is the total number of binding components present (whether masked or not) of size r, and B is the number of masker units present of size m. This model assumes that the maskers and binding components are independently inactivated by radiation and that upon a "hit" in a masked binding component the masker interacts with a free binding component. A model that assumes destruction of the masker when the binding component is hit would not lead to an increase in A above that at $D = 0$. In order to observe an increase in A, m must be greater than r.

At zero dose $A = A_T - B$. Since we normalize to A_0

$$A/A_0 = (A_T/A_0)e^{-rD} - (B/A_0)e^{-mD} \quad (2)$$

and at high doses the curve approaches $(A_T/A_0)e^{-rD}$. The difference curve is then

$$\frac{A_T}{A_0}e^{-rD} - \left(\frac{A_T}{A_0}e^{-rD} - \frac{B}{A_0}e^{-mD}\right) = \frac{B}{A_0}e^{-mD} \quad (3)$$

which is a single exponential whose slope reveals the molecular weight of the masker unit. The effect of salt on the TSH binding could equally well result from a change in the masker size as by a dissociation of masker units (m and B, respectively, in the equations). We tested for effects of the N component on the receptor as follows. When, prior to irradiation, membranes were incubated with Mg^{2+} and Gpp(NH)p (known modulators of TSH-responsive adenylate cyclase), TSH binding (measured by method 1, see Experimental Procedures) was qualitatively unchanged (data not shown). Binding to samples receiving 1.0 and 1.5 megarads was increased 30% relative to the unirradiated control; the slope at high doses was the same as the ground-state control, which was shown in Figure 1, suggesting no effect of these agents on the receptor. Attempts to determine whether the change in binding after irradiation resulted from a change in affinity or number of receptors were prevented by the poorly reversible nature of TSH binding (Kotani et al., 1975; Brennan et al., 1980).

Cholera Toxin Binding Component. The effect of radiation on the binding of cholera toxin is illustrated by Figure 3. Superimposed on the figure is the curve for inactivation of the TSH receptor, obtained by assay of the same membrane preparation. Low doses of radiation did not increase the specific binding of cholera toxin while they did augment the binding of TSH. Furthermore, the slopes at high doses of irradiation were markedly different. There was no statistically significant decrease in cholera toxin binding even after massive doses of ionizing radiation, indicating that the target size of the cholera toxin binding component is very small, perhaps less than 5000 daltons.

Adenylate Cyclase. The effect of ionizing radiation on ground-state adenylate cyclase is shown for the Mn^{2+} basal (Figure 4) and NaF-stimulated (Figure 5) activities. These inactivation curves are complex, resembling the biphasic curves obtained by Schlegel et al. (1979) in rat liver, and consistent with the presence of two components. From the slope at higher doses, the data yield M_r values of 85 000 ± 7000 for the basal and 140 000 ± 19 000 for the NaF-responsive adenylate cyc-

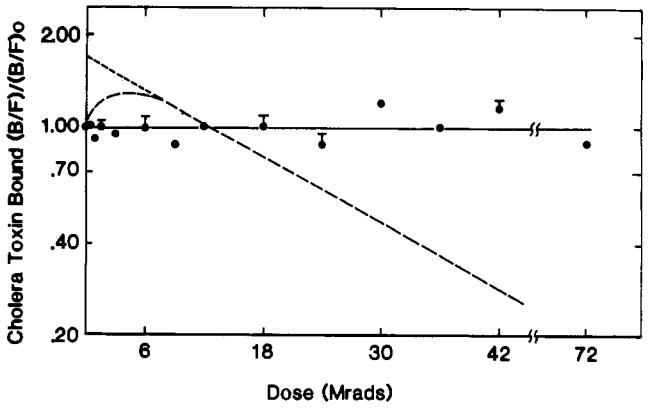

FIGURE 3: Radiation inactivation of the cholera toxin receptor. Samples of bovine thyroid plasma membranes were irradiated in duplicate at each dose indicated, then thawed, diluted, and assayed for cholera toxin binding [mean and range/2 (●)]. The data shown are representative of three membrane preparations irradiated on separate occasions. Superimposed on the figure is the curve of radiation inactivation of TSH binding to the same membrane preparation (--), see Figure 1.

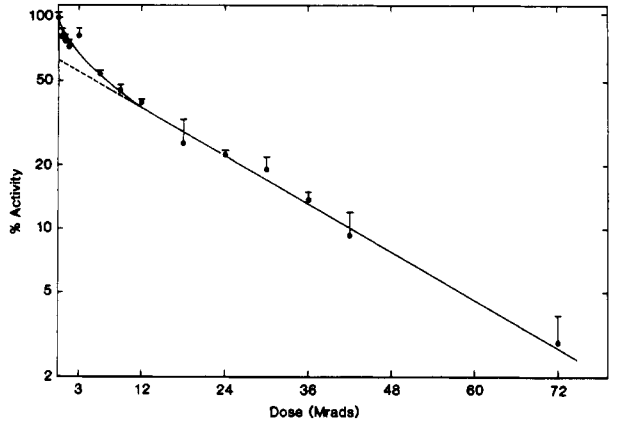

FIGURE 4: Radiation inactivation of ground-state membranes; assay of adenylate cyclase with Mn^{2+}. The data are normalized to a non-irradiated control of 202 ± 13 pmol of cAMP $(5\ min)^{-1}\ mg^{-1}$. Figures 4–6 are from the same experiment, with samples irradiated in duplicate followed by triplicate measurements of adenylate cyclase activity. The figures are representative of four (for basal and TSH response) or five (for NaF- or Gpp(NH)p-stimulated adenylate cyclase) experiments. The figures show the mean and range/2.

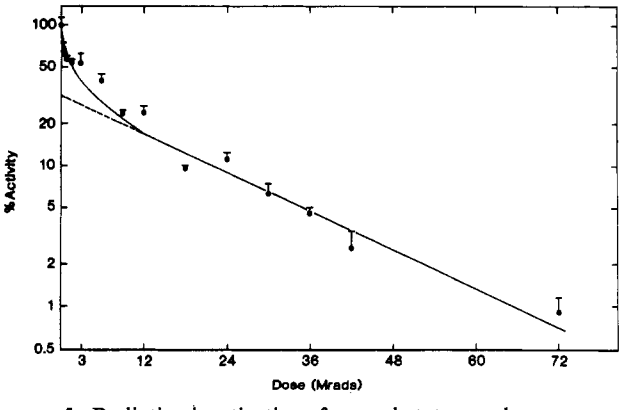

FIGURE 5: Radiation inactivation of ground-state membranes; assay of NaF-responsive adenylate cyclase. The activity of the nonirradiated controls was 1700 ± 200 pmol of cAMP $(5\ min)^{-1}\ mg^{-1}$.

lases (Table I). The data of the basal and NaF-stimulated activities at low radiation doses are consistent with the presence of larger components (150 000 daltons and greater than 10^6 daltons, respectively). Target-size analysis of Gpp(NH)p-responsive adenylate cyclase (Figure 6) resulted in a single

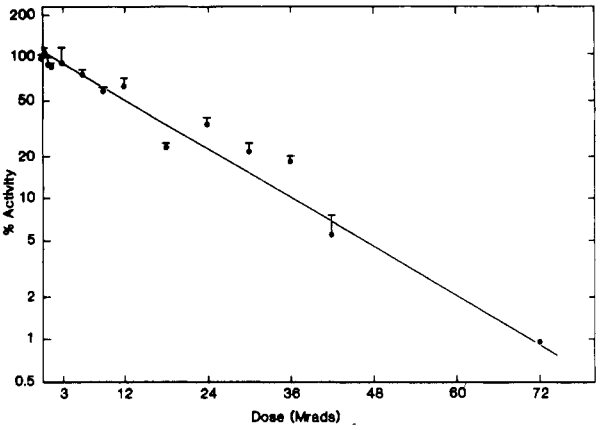

FIGURE 6: Radiation inactivation of ground-state membranes; assay of Gpp(NH)p-responsive adenylate cyclase. The activity of the nonirradiated controls was 520 ± 20 pmol of cAMP $(5\ min)^{-1}\ mg^{-1}$.

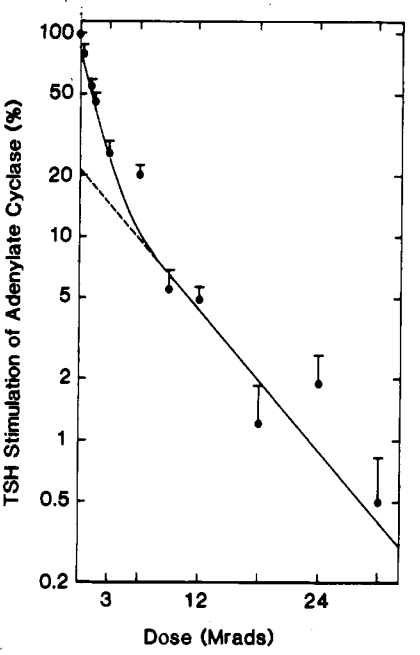

FIGURE 7: Radiation inactivation of ground-state membranes; assay of the stimulation of adenylate cyclase by TSH. The ordinate represents the difference between activity with TSH + Gpp(NH)p and activity with Gpp(NH)p alone normalized to the nonirradiated control value of 1589 ± 105 pmol of cAMP $(5\ min)^{-1}\ mg^{-1}$. Membrane samples were irradiated in triplicate at each dose, and each sample was assayed in triplicate.

target size of 150 000 ± 18 000 from the grouped data analysis (see Table I) or a similar value of 137 000 from the ungrouped data analysis. The target analysis of TSH stimulation of adenylate cyclase was obtained by difference of the activity with Gpp(NH)p alone (as in Figure 6) and the activity with TSH plus Gpp(NH)p (not shown) as described under Experimental Procedures. When these data were analyzed, a nonlinear curve was obtained (Figure 7). The regression lines of the ungrouped data from 0 to 3 megarads and from 9 to 30 megarads were significantly different ($p < 0.001$), suggesting the presence of at least two active components. The smaller of the components is calculated by the grouped analysis method to have a molecular weight of 240 000 and the larger a molecular weight greater than 10^6 (Table I).

Discussion

These experiments describe the target sizes of the TSH receptor and adenylate cyclase system in thyroid plasma membranes. Both the receptor and the hormone responsive

adenylate cyclase appear to be composed of multiple components. It may be useful to note that target analysis yields a measure of the size of the functional unit and not a particle size. The functional unit is the smallest unit capable of binding a ligand or of performing an enzymatic reaction (Kempner & Schlegel, 1979). In addition, as in this and other cases (Harmon et al., 1980), the size of the unit that masks or blocks this function can also be observed if the mass of the masker is sufficiently greater than the mass of the binding component. Hydrodynamic methods measure a particle structure that may include adherent components but do not necessarily measure the size of the functional unit.

TSH Receptor. We find that the radiation-inactivation curve of the TSH receptor is complex. The simplest model is a structure composed of a binding unit of 70 000 daltons and a large (>250 000-dalton) component(s) that mask(s) the binding. Previous evaluations of the size of the TSH receptor involved solubilization with lithium diiodosalicylate (Tate et al., 1975) and calculated the molecular weights of the peaks to be 280 000, 160 000, 75 000, and 15 000–30 000. Drummond et al. (1982) recently measured the Stokes radius of the TSH receptor–Triton X-100 complex to be 67 Å. This radius would correspond to a M_r of about 300 000 for a globular particle with little or no lipid or detergent bound or to a smaller asymmetric or detergent-rich particle similar to values reported by others (Dawes et al., 1978; Iida et al., 1981). One report suggests that the size of the holoreceptor for TSH is about 200 000 daltons and is composed of several subunits (Islam et al., 1983). However, the work is difficult to interpret because a valid measurement of molecular weight by this method requires that the SDS–polypeptide chain complex be free of disulfide cross-links (Nielsen & Reynolds, 1978). Preliminary observations on cross-linking TSH to the binding component have been made by using light-activated cross-linkers followed by SDS gel electrophoresis in the presence of reducing agents (Eggo et al., 1981; McQuade et al., 1983; T. B. Nielsen, M. S. Ferdows, and J. B. Field, unpublished observations). These studies suggest that the polypeptide size of the TSH–receptor complex may be about 65 000, 70 000, or 84 000 daltons. Taken together, these observations suggest a TSH receptor composed of two macromolecular parts: a TSH binding component of about 70 000 daltons and other components that would increase the hydrodynamic size and possibly mask the TSH binding activity.

An important adjunct to the measurement of binding is the measurement of function. Comparison of the size of the structures necessary for TSH stimulation of adenylate cyclase (240 000 ± 28 000 daltons) with that necessary for Gpp(NH)p-stimulated adenylate cyclase (150 000 ± 18 000 daltons) reveals a difference (about 90 000 daltons) that should correspond to the physiological receptor. This value appears to be similar to that calculated from the binding data. This suggests that the 70 000-dalton TSH binding component observed by target analysis may be responsible for the activation of adenylate cyclase.

This study suggests the presence of a component of M_r >250 000 in the thyroid plasma membranes that masks the binding of TSH. This observation is consistent with several reports that TSH binding is increased after treatments that affect the membrane (Davies et al., 1977; Pekonen & Weintraub, 1980; Manley & Bourke, 1981; Petersen et al., 1979). Such reports are commonly expressed in terms of "high-affinity" or "low-affinity" binding, although the tight, poorly reversible binding of TSH to the receptor (Kotani et al., 1975; Brennan et al., 1980) precludes a stringent analysis by the method of Scatchard. The effects of these agents may correspond to release of the binding component from the influence of a macromolecular inhibitor. NaCl (50 mM) diminishes the relative amount of TSH bound to the low-affinity but not the high-affinity site (Pekonen & Weintraub, 1979). Thus, the fact that 50 mM NaCl did not diminish the influence of the putative inhibitor suggests that the masker modulates the high-affinity receptor, which can couple to the adenylate cyclase (Saltiel et al., 1980).

A large binding inhibitor has also been described as a part of the insulin receptor (Harmon et al., 1980). In that system, a role in modulation of binding was postulated for the inhibitor. The large component in thyroid membranes may serve a similar function.

The simplest model that is consistent with all of these TSH receptor studies is that of a collection of free binding units (r, 70 000 daltons each) and an approximately equal number of larger complexes ($r + m$, >300 000 daltons each). The larger units contain a masked binding unit (70 000 daltons) that can be expressed after radiation destruction of other portions (>240 000 daltons) of the complex. Is this larger complex the same as that observed by others (Drummond et al., 1982; Iida et al., 1981; Dawes et al., 1978; Tate et al., 1975) during purification of the solubilized receptor? Does the >240 000-dalton "masking" unit contain one or more guanine nucleotide regulatory complexes (60 000 daltons each, vide infra), and if so, are catalytic units also part of the complex?

Although similarities between the TSH receptor and cholera toxin receptor have been reported (Kohn, 1978), this work suggests some fundamental differences between the two. Cuatrecasas (1973) found that cholera toxin bound to ganglioside GM1, consistent with our observation of the size (less than 5000 daltons) of the cholera toxin binding component but very different from the size of the TSH receptor (Table I). Recent findings (Pekonen & Weintraub, 1980; Holmes et al., 1980; Ross et al., 1979; Van Sande et al., 1979) including those by Beckner et al. (1981) who determined the lack of complex gangliosides in a cultured thyroid cell line also suggest that complex gangliosides are not necessary for TSH function.

Adenylate Cyclase. The M_r 85 000 component of thyroid adenylate cyclase responsible for basal (Mn^{2+}) activity is similar in molecular weight to that of turkey erythrocyte (92 000) (Nielsen et al., 1981) and the calculations for thyroid (80 000) by Goldhammer et al. (1980) but smaller than those from rat liver (150 000) (Schlegel et al., 1979) or S49 murine *cyc*$^-$ lymphoma (190 000) (Ross et al., 1978). The hydrodynamic measurement of 190 000 daltons for the C in S49 *cyc*$^-$ is difficult to interpret because of recent observations (Hildebrand et al., 1982; Jakobs et al., 1983) that this membrane contains an inhibitory N component that may interact with C. In thyroid, the 85 000-dalton structure appears to be the minimum capable of sustaining adenylate cyclase activity and probably corresponds to the functional catalytic unit. An adenylate cyclase from testis is 56 000 daltons, but this enzyme is water-soluble and does not respond to hormones (Neer, 1978).

Both these (Table I) and previous (Asbury et al., 1978; Goldhammer et al., 1980) measurements of the thyroid adenylate cyclase suggests a smaller size N component (55 000–70 000 daltons) than was found by target analysis for turkey erythrocytes (130 000 daltons) (Nielsen et al., 1981) and rat liver (>80 000 daltons) (Schlegel et al., 1979) or by hydrodynamic means in S49 lymphoma (130 000 daltons) (Howlett & Gilman, 1980) and human erythrocytes (126 000 daltons) (Kaslow et al., 1980). The nature of the large components

(>10^6 daltons) observed with the thyroid adenylate cyclase assayed with NaF or TSH is a matter of interest especially because large components were also observed in adenylate cyclases from rat liver (Schlegel et al., 1979) and adipocyte (Schlegel et al., 1980) but not turkey erythrocyte (Nielsen et al., 1981). Schlegel et al. (1979) suggested that large components in liver may be oligomeric forms of the smaller units, and we are currently investigating this possibility for thyroid cyclase. These large components may reflect the involvement of an inhibitory N component in the action of hormone and in the stimulation by NaF but not in the stimulation by Gpp(NH)p. The 150000-dalton component II observed with MnATP as substrate probably corresponds to a contribution by the N component because a small stimulation by endogenous guanine nucleotides would be expected under these experimental conditions (Totsuka et al., 1982).

In conclusion, the structures of both the physiological TSH receptor and the adenylate cyclase in thyroid plasma membranes appear to be complex, with both large and small macromolecular components present. These components in the receptor may be involved in regulation of its function or in an effector system. The large components observed in the adenylate cyclase probably reflect the association of the small components of the system. It is as yet unknown whether the structures in the isolated membranes also exist in the intact cell. If so, this suggests a high level of regulation.

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Oligomeric Structure of Molybdate-Stabilized, Nontransformed 8S Progesterone Receptor from Chicken Oviduct Cytosol†

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ABSTRACT: Bioaffinity-purified, molybdate-stabilized, nontransformed 8S progesterone receptor (8S-PR) of chicken oviduct cytosol has been analyzed first by ion-exchange chromatography, second by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as revealed by silver staining and immunoblotting with anti-8S-PR and anti-B-PR-subunit polyclonal antibodies, and third by progestagen affinity labeling and cross-linking with glutaraldehyde. Two forms (I and II) of 8S-PR were detected as described by Puri and colleagues [Puri, R. K., Grandics, P., Dougherty, J. J., & Toft, D. O. (1982) *J. Biol. Chem. 257*, 10831–10837]. They contained the non-progestin-binding 90-kDa protein [Joab, I., Radanyi, C., Renoir, M., Buchou, T., Catelli, M. G., Binart, N., Mester, J., & Baulieu, E. E. (1984) *Nature (London) 308*, 850–853] and progesterone-binding proteins described by Schrader and colleagues [Schrader, W. T., Birnbaumer, M. E., Hughes, M. R., Weigel, N. L., Grody, W. W., & O'Malley, B. W. (1981) *Recent Prog. Horm. Res. 37*, 583–599], that is, A (~79 kDa) in form I or B (~110 kDa) in form II. Cross-linked 8S-PR molecules (I and II) displayed an ~8S sedimentation coefficient and ~7.0-nm Stokes radius, similar to the values reported for non-cross-linked purified 8S-PR, and the mass was found to be 260 kDa by SDS–PAGE. From these results, from specific activity measurements of purified 8S-PR, and from densitometric scanning after SDS–PAGE of 8S-PR I and II, we propose that each molecule of 8S-PR includes one molecule of the hormone-binding subunit A or B and two molecules of the non-hormone-binding 90-kDa protein. The biological significance of the 8S-PR structures purified from the oviduct cytosol is discussed with reference to recent immunohistochemical findings indicating the simultaneous presence of progesterone-binding and non-progesterone-binding components of progesterone receptor in target cell nuclei [Gasc, J. M., Renoir, J. M., Radanyi, C., Joab, I., Tuohimaa, P., & Baulieu, E. E. (1984) *J. Cell Biol.* (in press)].

Two high-affinity progesterone-binding proteins denoted A and B have been described in the chick oviduct cytosol (Schrader & O'Malley, 1972; Kuhn et al., 1975; Coty et al., 1979; Gronmeyer et al., 1983; Renoir et al., 1984). Their characterization and purification have been accomplished under conditions that lead to the "activation" or "transformation" of the progesterone receptor (PR), such as ammonium sulfate precipitation (Schrader et al., 1977) or exposure to high salt (Renoir et al., 1984). However, all specific high-affinity progestin-binding constituents of low-salt cytosol sediment as 8S species (Toft & Nishigori, 1979; Wolfson et al., 1980). It has been proposed that the 8S molecules (8S-PR)[1] represent the "native" PR in the absence of hormone (Baulieu et al., 1983), that is, a "nontransformed" form of the receptor showing low affinity for polyanions (Nishigori & Toft, 1980; Wolfson et al., 1980). Under cell-free conditions, 8S-PR dissociates into 4S-transformed or activated PR forms from which progestin dissociation rates are slower than from 8S-PR and which bind strongly to polyanions [Wolfson et al., 1981; see Moudgil (1983) for a recent review].

By use of the stabilizing property of molybdate ions, 8S-PR was recently purified from chick oviduct cytosol (Renoir et al., 1982a; Puri et al., 1982). Coomassie Blue staining of disc SDS–PAGE revealed a 90-kDa protein as its main constituent (Renoir et al., 1982a; Puri et al., 1982). Immunological and bioaffinity chromatography studies (Joab et al., 1983, 1984;

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[1] Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 8S-PR, nontransformed molybdate-stabilized 8S progesterone receptor; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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