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ANTIBODY AGAINST NUCLEAR THYROID HORMONE RECEPTORS*

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Rat liver nuclear thyroid hormone receptor was purified to 700-1600 pmol T_3 binding capacity/mg protein by sequentially using hydroxylapatite column, ammonium sulfate precipitation, Sephadex G-150 gel filtration, DNA-cellulose column, DEAE-Sephadex A-50 column, and heparin-Sepharose column. Serum from a mouse immunized using this purified receptor preparation caused a shift of $[^{125}I]T_3$-receptor peak on glycerol density gradient sedimentation from 3.4 S to approximately 7 S. $[^{125}I]T_3$-receptor complex was immunoprecipitated using this serum and goat anti-mouse IgG. The serum showed reduced ability to immunoprecipitate the globular T_3 binding fragment with Stokes radius of 22 Å produced by trypsin digestion, a receptor fragment which has core histone and hormone binding but not DNA binding activity. These data indicate the production of anti-nuclear thyroid hormone receptor antibody which mainly recognized epitopes unrelated to hormone and core histone binding domain.

Radioactive thyroid hormone has been the only marker for studying the nuclear thyroid hormone receptor protein, which is considered to mediate most of the biological responses of thyroid hormone (1, 2). Production of an antibody to the protein would allow new approaches to the study of the receptor. The major problem in raising antibodies to the receptor has been the extreme difficulty in purification of the protein.

Recently we reported purification of nuclear thyroid hormone receptor yielding preparations having 0.2 nmol 3,5,3'-triiodo-L-thyronine (T_3) binding capacity in a single purification, with a final purity of 904 pmol T_3 binding capacity/mg protein (3). Purified receptor showed high affinity binding for T_3, relative affinity for iodothyronine analogues identical to those seen

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using crude nuclear extracts, estimated molecular weight of 49,000 by the hydrodynamic method and $NaDodSO_4$-polyacrylamide gel electrophoresis, and a change in molecular size after partial proteolysis identical to crude receptor, all of which suggest that we have purified the classical nuclear thyroid hormone receptor without changing its characteristics.

We now describe the production of antisera from a mouse immunized using purified nuclear thyroid hormone receptor.

METHODS

L-T_3 and soybean trypsin inhibitor were purchased from Sigma. Dithiothreitol (DTT) was from Bethesda Research Laboratory. [^{125}I]T_3 (3400 μCi/ μg) was from New England Nuclear. Goat antiserum against mouse IgG (heavy chain and light chain specific) was from Calbiochem-Behring.

Purification of Nuclear Thyroid Hormone Receptor

Rat liver nuclear thyroid hormone receptor was purified as previously described (3) with minor modifications as follows. Soybean trypsin inhibitor (0.2 μg/ml) was added at the time of tissue homogenization and treatment of crude nuclei with Triton X-100. Elution of receptor from the final Heparin-Sepharose column was performed using 0.7 M NaCl, 2 mM EDTA, and 20 mM potassium phosphate buffer, pH 8.0. T_3 binding capacity was determined after each column procedure as previously described (3).

Immunization Method

Receptor eluted from Heparin-Sepharose column was lyophilized. A minimal amount of double distilled water was added to dissolve protein and salt. Protein was precipitated by 60% saturation of ammonium sulfate and the pellet was dissolved in phosphate buffered saline, pH 7.4 (PBS). Female mice at 5 weeks of age were injected with freshly prepared receptor in Freund's complete adjuvant (initial two injections) or in Freund's incomplete adjuvant, subcutaneously at multiple sites (more than 10 sites) on the back.

Glycerol Density Gradient Sedimentation

Ten to seventy μl of serum from immunized mice or non-immunized mice were incubated with 0.08 pmol [^{125}I]T_3-receptor complex deprived of free [^{125}I]T_3 by Dowex resin treatment (4) in 0.3 M KCl, 1 mM $MgCl_2$, and 10 mM potassium phosphate buffer, pH 8.0, in a total volume of 100 μl at 0-4 C for 3h. At the end of incubation, 1 mg ovalbumin was added and samples were analyzed by glycerol density gradient sedimentation as described previously (5).

Immunoprecipitation Assay

Three μl of serum from immunized mice or non-immunized mice and 0.01 pmol [^{125}I]T_3-receptor deprived of free hormone in a final volume of 100 μl of PBS were incubated overnight at 0-4 C. Twenty μl of properly diluted goat antiserum to mouse IgG was added and incubation was continued for an additional 4 h. The dilution of the second antibody, required to precipitate all mouse immunoglobulins, was determined in advance. After incubation, the samples were centrifuged. The pellets were washed twice with PBS and radioactivities were counted. During the incubation period, 87 ± 7% (mean ± SD of six determinations) of [^{125}I]T_3 remained bound to the receptor.

Protein content was estimated by a Coomassie blue method (6) using a bovine serum albumin standard. Protein determinations of receptor preparations using a modified Lowry method (7) or a Biuret reaction (8) gave similar results.

RESULTS

Purification of Receptor and Immunization

The typical result of receptor purification is shown in Table 1. 0.24 - 0.53 nmol T_3 binding capacity was recovered in 0.33 - 0.55 mg total protein. Table 2 shows the immunization protocol for a mouse which developed a positive anti-nuclear thyroid hormone receptor antibody. In total 1.07 nmol of receptor was injected during an immunization period of 42 days (4 injections at 2 week intervals). Serum was obtained 10 days and 20 days after the final injection and used in this study.

Sedimentation of Antibody-receptor Complex

The formation of antibody-receptor complex was analyzed by glycerol density gradient sedimentation. As shown in Fig. 1, serum from an immunized mouse caused partial shift of the $[^{125}I]T_3$-receptor peak from 3.4 S to approximately 7 S. Formation of the 7 S peak was more prominent using 30 µl immune serum than using 10 µl serum. Up to 70 µl serum from a control mouse did not cause any change in the 3.4 S $[^{125}I]T_3$-receptor peak.

TABLE 1

PURIFICATION OF NUCLEAR THYROID HORMONE RECEPTOR

	Purity (pmol T_3/mg protein)	Recovery of T_3 binding capacity (nmol)	Total protein (mg)
NE	1.7	5.71	3420
HAP	8.1	4.81	594
Seph G-150	43.8	2.18	49.8
DNA-Cell	524	0.91	1.74
DEAE-Seph	768	0.83	1.08
Hep-Seph	870	0.37	0.43

NE; nuclear extract. HAP; hydroxylapatite column. Seph G-150; Sephadex G-150 column. DNA-cell; DNA-cellulose column. DEAE-Seph; DEAE-Sephadex A-50 column. Hep-Seph; Heparin-Sepharose column.
T_3 binding capacity was determined by incubating receptor preparations with 5 nM $[^{125}I]T_3$ in the absence (total T_3 binding) and presence (nonspecific T_3 binding) of 300 nmol unlabeled T_3.
Specific T_3 binding was calculated by subtracting nonspecific T_3 binding from total T_3 binding.

TABLE 2

IMMUNIZATION PROTOCOL

Immunization	Days	Receptor injected (nmol T_3 binding)	Protein (mg)	Adjuvant
1	0	0.31	0.38	FCA
2	11	0.22	0.26	FCA
3	28	0.25	0.24	FIA
4	42	0.29	0.34	FIA

FCA; Freund's complete adjuvant FIA; Freund's incomplete adjuvant

Immunoprecipitation Assay

Results in Table 3 shows that the $[^{125}I]T_3$-receptor complex was precipitated using immune serum and goat antiserum to mouse IgG (heavy and light chain specific). Significantly more $[^{125}I]T_3$-receptor complex was precipitated using immune serum (17.7% of total $[^{125}I]T_3$-receptor complex added) than using control serum. Several control serum from non-immunized mice and immunized mice without anti-receptor antibody showed 1.5 - 2.8% of total

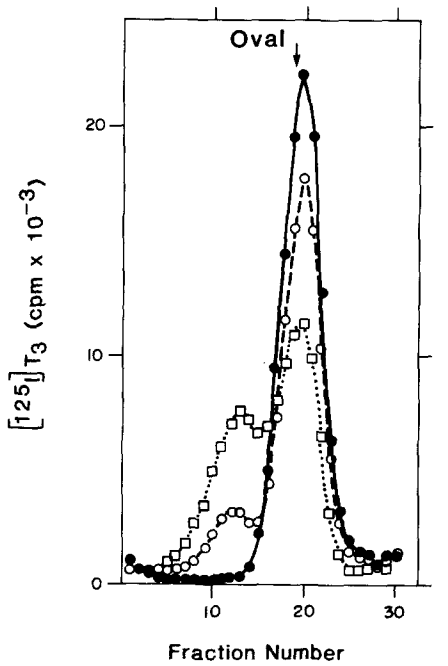

Figure 1. Glycerol density gradient sedimentation. 0.08 pmol $[^{125}I]T_3$-receptor incubated with 50 µl control serum (●——●), 10 µl (O------O) or 30 µl (□ ····· □) of immune serum was analyzed by glycerol density gradient sedimentation. ↓ ; ovalbumin.

TABLE 3

IMMUNOPRECIPITATION OF $[^{125}I]T_3$-RECEPTOR COMPLEX

	Immunoprecipitated $[^{125}I]T_3$-Receptor (fmol)	
	Control serum	Immune serum
Native $[^{125}I]T_3$-receptor	0.22 ± 0.05 †	1.77 ± 0.05 *
22 Å-$[^{125}I]T_3$-receptor	0.19 ± 0.06 †	0.40 ± 0.02 *

Immunoprecipitable $[^{125}I]T_3$-receptor complex was determined using control serum or immune serum and 10 fmol of native $[^{125}I]T_3$-receptor complex or 22 Å-tryptic $[^{125}I]T_3$-receptor fragment as described in Methods.

† Mean ± SD of results from 9 mice (4 non-immunized mice and 5 immunized mice, which were negative for anti-receptor antibody by density gradient sedimentation analysis)

* Mean ± range of duplicate determinations.

22 Å-$[^{125}I]T_3$-receptor was produced by digesting $[^{125}I]T_3$ labeled nuclear extract with 5 µg trypsin/mg protein as previously reported (9).

$[^{125}I]T_3$-receptor recovery in the immunoglobulin pellet. Immune serum did not react with free $[^{125}I]T_3$. In order to determine epitopes recognized by immune serum, $[^{125}I]T_3$-receptor complex was subjected to partial tryptic digestion, which produced a $[^{125}I]T_3$-receptor complex with Stokes radius of 22 Å measured by Sephadex G-150 gel filtration. Previous studies showed that this tryptic T_3 binding fragment is a globular protein with molecular weight of 26,000 - 27,000 (3, 9) which showed core histone and hormone binding activity identical to native receptor but which lost DNA binding activity completely (9). The immune serum had decreased reactivity with the tryptic $[^{125}I]T_3$-receptor fragment, but showed significantly higher reactivity than did a control serum.

DISCUSSION

This paper presents the first demonstration of anti-nuclear thyroid hormone receptor antibody in serum from a mouse immunized with highly purified rat liver receptor. Antibody-receptor complex was demonstrated by the shift of $[^{125}I]T_3$-receptor peak to a heavier sedimenting unit on density gradient sedimentation, and by immunoprecipitation of the $[^{125}I]T_3$-receptor complex. Decreased reactivity of antiserum with the 22 Å-tryptic $[^{125}I]T_3$-receptor fragment (Table 3) indicates that most of the antibodies present in the immune

serum recognize epitopes unrelated to hormone and core histone binding domain of receptor, since the 22 Å-tryptic [^{125}I]T_3-receptor fragment has no DNA binding activities but retains hormone and core histone binding (9). However, the fact that the immune serum showed significant immunoprecipitation of 22 Å-[^{125}I]T_3-receptor fragment suggests that some subset of antibodies recognized the 22 Å-[^{125}I]T_3-receptor fragment because of polyclonality of anti-receptor antibodies present in the immune serum. It is interesting to note that most of the antibodies raised against glucocorticoid hormone receptor also react with an epitope which is lost when examined using hormone receptor complex after removal of the DNA binding domain of receptor by proteolysis (10, 11). Carlstedt-Duke et al (11) suggested there is a third domain in the glucocorticoid receptor, distinct from hormone binding domain and DNA binding domain using antibodies. In order to further demonstrate the epitopes distinct from hormone and core histone binding domain of thyroid hormone receptor, epitope mapping study using monospecific antibody is required. Such a study would clearly discriminate this possibility from the theoretical possibility that reduced recognition of 22 Å-[^{125}I]T_3-receptor complex by antiserum is merely due to conformational change in the receptor molecule created by trypsin cleavage so that most of the epitopes present in the hormone and core histone binding domain are not capable of binding antibodies. Since protein samples used for immunization are extremely heterogeneous, the antisera obtained are clearly polyspecific. To obtain antibodies useful for various studies, including epitope mapping, immunoaffinity purification of receptor, and ligand independent immunological assay of receptor, the application of monoclonal antibody technique is useful to obtain monospecific antibodies uncontaminated with other immunoglobulins.

The present study clearly shows that the receptor protein itself is immunogenic, and our purified receptor can be utilized to elicit a specific immune response, therefore demonstrating the feasibility of obtaining monoclonal antibodies against nuclear thyroid hormone receptor.

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