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Antibodies against the human cellular 3,3′,5-triiodo-L-thyronine-binding protein (p58)

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High-titer antibodies against a cellular thyroid hormone-binding protein (M_r 58000, p58) were developed by a special immunization method. To enhance immune responses, this method uses a boosting protocol in which repeated injections of small amounts of antigen are administered at 2-day intervals. Antibodies were detected 1 week after the last injection of antigen by ELISA, Western dot blotting and immunoprecipitation. The anti-p58 antibodies recognize p58 which is bound to the thyroid hormone. With the availability of anti-p58 antibodies, it has become possible to study cellular localization and function.

Immunization; ELISA; Western blotting; Metabolic labeling; Thyroid hormone; T_3-binding protein

1. INTRODUCTION

Recent studies have indicated that the entry of the thyroid hormone 3,3′,5-triiodo-L-thyronine (T_3) into cells is a receptor- or carrier-mediated process. This process has been extensively characterized in isolated hepatocytes [1–3] and cultured cells [4–6]. The results indicate that a cellular protein is involved in T_3 uptake.

Recently, we have isolated a cellular T_3-binding protein of M_r 58000 (p58). This protein has now been purified to apparent homogeneity and retains its T_3-binding activity [6]. In order to determine its intracellular location and role in thyroid hormone transport, and to clone its gene, specific antibodies against p58 would be useful. However, attempts to develop antibodies using conventional immunization protocols met with failures. The present work describes a special method which gives high-titer antibodies against p58. Furthermore, the level of antibodies was found to be maintained for up to 5 months.

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Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PBS, phosphate-buffered saline

2. MATERIALS AND METHODS

[3,3′,5-^{125}I]T_3 (2200 Ci/mmol) was from New England Nuclear, CHAPS from Sigma and goat anti-mouse immunoglobulin from Cappel. The avidin/biotin/avidin-horseradish peroxidase system (ABC kit) was from Vector Laboratories. A431 cells were propagated in Dulbecco-Vogt's medium containing 10% fetal calf serum as in [7].

2.1. *Immunization*

Native p58 was purified as in [6]. 5 μg purified p58 was mixed with an equal volume of complete adjuvant and half was injected into mice intraperitoneally and half subcutaneously. 3 weeks later, 2 μg purified p58 with incomplete adjuvant was introduced similarly. 2 days later, 2 μg p58 in 200–500 μl PBS was injected intraperitoneally. The injection was repeated for an additional four times at intervals of 2 days. Sera were screened 1 week after the last boost.

SDS-denatured and hemocyanin-conjugated p58 were also used as immunogens. SDS-denatured p58 was obtained by elution of p58 from SDS-polyacrylamide gels as described [7]. Conjugation of p58 to hemocyanin was carried out using 2-iminothiolane as a cross-linking reagent according to [8].

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2.2. *ELISA assay*

Assays were carried out at 22°C. Purified p58 (0.1 μg/40 μl of 0.5 mM CHAPS in 80 mM phosphate buffer) was added to each well in 96-well microtiter plates (Dynetech). Blocking, washing and reaction with substrate were performed as in [9].

2.3. *Western dot blotting*

p58 (0.05 μg) was spotted onto nitrocellulose paper. After drying, p58 was reacted with immune serum in various dilutions. The ABC kit was used to identify positive serum as described in [9].

2.4. *Metabolic labeling and immunoprecipitation*

A431 cells were labeled with [^{35}S]methionine for 20 h. CHAPS extractions of cells and immunoprecipitation were carried out as in [10].

2.5. *Immunoprecipitation of [^{125}I]T$_3$-bound p58*

Binding of p58 with [^{125}I]T$_3$ was carried out as described [6]. The [^{125}I]T$_3$-bound p58 was immunoprecipitated by incubation with 10 μl anti-p58 antiserum in 0.5 ml binding buffer at 4°C for 30 min. Goat anti-mouse immunoglobulin (1.79 mg in 100 μl binding buffer) was added. After incubation for 30 min at 4°C, the immune complex was pelleted by centrifugation at 11000 × *g* for 15 min. Radioactivity was determined by a γ-counter.

3. RESULTS

3.1. *Immunization*

Table 1 lists the immunogens which have been used in attempts to elicit antibodies against human p58. Native SDS-treated and hemocyanin-conjugated p58 were used as immunogens. The amounts of modified and unmodified immunogen used were similar. However, except for one in which a special protocol was used in the second boost, all failed to elicit antibody production. When native p58 was injected repeatedly at 2-day intervals into five mice, anti-p58 antibodies were detectable in three mice 1 week after the final injection. The two remaining mice also developed anti-p58 antibodies 2 weeks later. The same protocol was repeated in another experiment; high-titer antibodies were detected in all five mice 1 week after the last administration of antigen. The antibody levels were monitored periodically and found to be maintained at high levels for up to 5 months.

Table 1

Immunization with p58

Immunogen	Primary injection[a] (μg)	First boost[b] (μg)	Second boost[b] (μg)
SDS-treated p58	5	5	5
SDS-treated p58 conjugated to hemocyanin	5–10	5–10	5–10
Native p58 conjugated to hemocyanin	5	5	5
Native p58	5	2	2[c]

[a] Complete adjuvant

[b] Antigen was mixed with incomplete adjuvant and injected 3 weeks after primary injection

[a,b] Half of the antigen was administered intraperitoneally and half subcutaneously

[c] Boosting was repeated 5 times at an interval of 2 days without adjuvant. Antigen was injected intraperitoneally

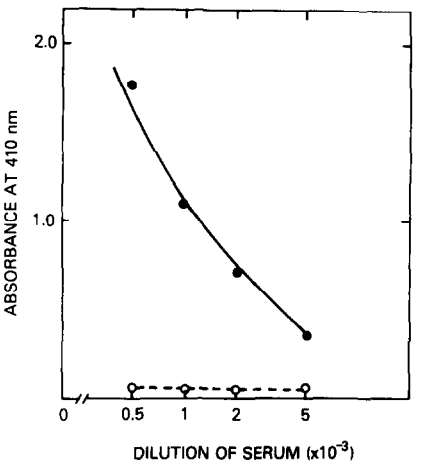

Fig.1. Titration of anti-p58 antiserum by ELISA assay. (●—●) Immune serum, (○---○) preimmune serum.

3.2. *Detection of anti-p58 antibodies*

Screening of anti-p58 antibodies in mice was carried out using the following methods. (i) ELISA assay. Fig.1 shows the concentration dependence of the immunoreactivity of mouse no.3 antiserum. At 1:5000 dilution of serum, the antibody is still detectable, whereas the preimmune serum yielded only the background reading. The sera from the other four mice have similar titers. (ii) Western dot blotting. Fig.2 shows the concentration dependence of the immunoreactivity of the serum from mouse no.3 where 50 ng purified p58 is spotted onto nitrocellulose. The reactivity is concentration dependent and detectable at 1:500 dilution. (iii) Immunoprecipitation of [^{35}S]methionine-labeled cellular extracts. Fig.3 shows the

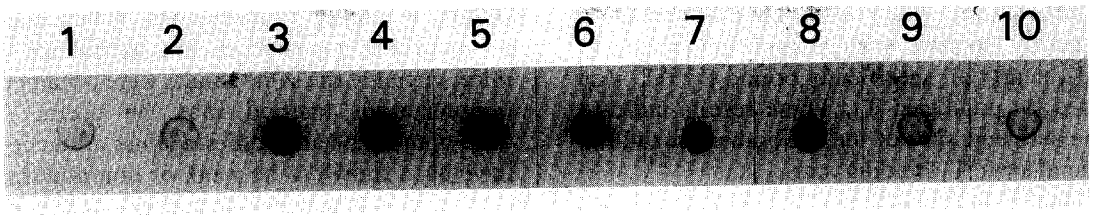

Fig.2. Titration of anti-p58 antiserum by Western dot blotting. Purified p58 (0.05 μg) was spotted onto nitrocellulose paper and reacted with increasing dilutions of sera. (1–2) Preimmune serum, (3–10) immune serum; serum dilution (1–4) 1:10, (5,6) 1:100, (7,8) 1:500, (9,10) 1:1000.

autoradiogram of immunoprecipitable protein bands from mouse no.3 antiserum at three dilutions. Compared to lane 1 in which preimmune serum was used, lanes 2–4 showed three specific bands of 91, 58 and 38 kDa, respectively. The 58 and 38 kDa bands are the most intense. The 38 kDa protein could be the degradation product of p58. Since this is a polyclonal antibody, it is not unexpected to find minor contaminating proteins, such as 91 kDa protein, being co-immunoprecipitated. Comparing lanes 2 and 3, less p58 is immunoprecipitated at 1:50 dilution than at 1:500, indicating that the immunoglobulins exceed the binding capacity of *Staphylococcus aureus*. On further dilution of serum, immunoprecipitation of the p58 band became concentration-dependent. (iv) Immunoprecipitation of $[^{125}I]T_3$-bound p58. To determine whether the antigenic sites are directed against the binding of T_3 to p58, p58 prebound to $[^{125}I]T_3$ was immunoprecipitated using goat anti-mouse IgG as second antibody. As shown in table 2, nearly 2-fold more $[^{125}I]T_3$ bound to p58 was immunoprecipitated as compared to

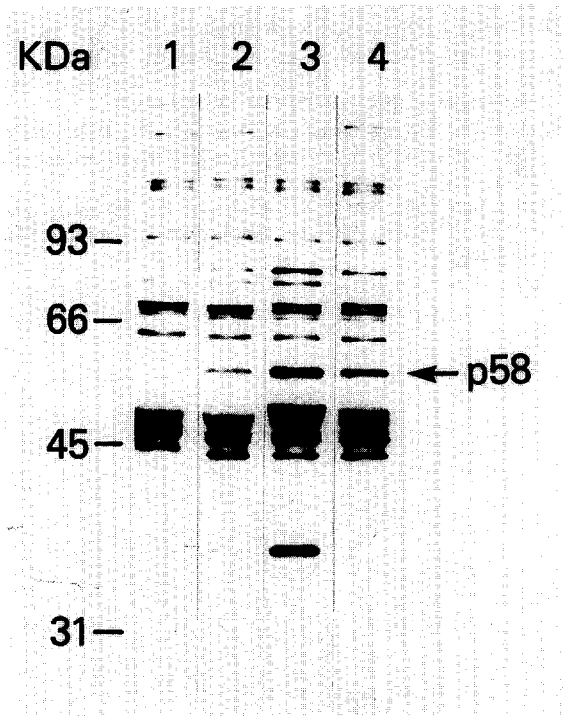

Fig.3. Autoradiogram of the immunoprecipitates from [^{35}S]methionine-labeled cellular extracts by anti-p58 antiserum. A431 cells (5 × 10^6 cells/100 mm dish) were labeled with [^{35}S]methionine (1 mCi) for 20 h at 37°C. Cells were extracted using CHAPS and immunoprecipitated with a 1:50 dilution of preimmune serum (lane 1) and 1:50 (lane 2), 1:500 (lane 3) and 1:5000 (lane 4) dilutions of immune serum.

Table 2

Immunoprecipitation of $[^{125}I]T_3$-bound p58

Serum	$[^{125}I]T_3$-bound p58 antibody complex[a] (dpm × 10^{-3})	
	–	Dissociation with unlabeled T_3[b]
Immune	8.9	3.3
Preimmune	4.5	3.2

[a] Purified p58 (1.9 μg) was incubated with 2 nM $[^{125}I]T_3$ in 500 μl binding buffer at 4°C for 30 min. The $[^{125}I]T_3$-bound p58 was separated from free $[^{125}I]T_3$ as described in section 2. $[^{125}I]T_3$-bound p58 was divided into two equal aliquots and incubated with 10 μl immune or preimmune serum for 30 min. Goat anti-mouse immunoglobulin (1.8 mg) was used to precipitate $[^{125}I]T_3$-bound p58 immune complex as described in the text

[b] The immune complex obtained by centrifugation was resuspended in binding buffer (500 μl) in the presence or absence of 20 μM T_3 for 30 min. The immune complex was pelleted by centrifugation at 11000 × *g* for 10 min and counted

preimmune serum. When the immune complex was subsequently treated with excess unlabeled T_3 (20 μM), nearly all specifically bound [^{125}I]T_3 was displaced by unlabeled T_3. These results indicate that the antigenic sites of the antibodies are unlikely to be against T_3-binding sites.

4. DISCUSSION

This paper describes the development of antibodies against p58, a cellular thyroid hormone-binding protein. The specificity was demonstrated by ELISA, Western dot blotting and immunoprecipitation of [^{35}S]methionine-labeled protein bands. The antigenic sites of the polyclonal antisera are not against thyroid hormone-binding sites. Furthermore, [^{125}I]T_3 in the immune complex can be displaced by unlabeled T_3.

p58 is not a very immunogenic protein. Many earlier attempts using standard immunization methods all failed to elicit specific antibodies against p58. Native and SDS-denatured p58 was conjugated to hemocyanin in the hope of enhancing its immunogenicity (table 1). However, no anti-p58 was detected. It was believed that the failure to elicit immune responses could be due to the instability of p58. An immunization protocol with repeated injections at intervals of 2 days was finally tried and proved to be successful. This procedure is highly reproducible. When the same protocol was repeated in the second set of five mice, virtually the same results were obtained. This immunization method may be useful in the development of antibodies against antigens which have been shown to be weak or nonimmunogenic.

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