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Characterization of a major development-regulated serum thyroxine-binding globulin in the euthyroid mouse

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We confirm our finding of a major development-regulated thyroxine-binding globulin (TBG) in the serum of the euthyroid mouse and investigate a number of its binding, structural and regulatory properties. Between 16 days foetal and 60 days postnatal life, the thyroxine (T_4)- and tri-iodothyronine (T_3)-binding activities of the sera show a striking ontogenic pattern: the binding is 2–3 times higher in foetuses than in mothers, then further increases after birth, reaching between 3 and 5 days maximum values which are 7–8 times higher than the adult ones. This pattern is not correlated with the ontogenesis of the acknowledged specific (transthyretin, TTR) and non-specific (albumin, α_1-foetoprotein) thyroid-hormone carriers of the mouse sera. PAGE studies demonstrate that the protein responsible for the elevated binding of the perinatal period is an α_1-globulin, with a migration similar to that of human and rat TBGs. Scatchard analysis is consistent with the notions that the T_4-binding sites of TBG have high association constants, about two orders of magnitude above the T_4 sites of TTR (10^9 M^{-1} as against 10^7 M^{-1}) and low capacities (37 and 4 nmol/g of serum proteins in pups and adults respectively). Isoelectric focusing (i.e.f.) demonstrates that mouse TBG is a microheterogeneous protein separable, as a function of the pH gradient, in up to 10–12 isoforms, Marked shifts of the relative abundance of isoforms in the course of development are evidenced. The modulation of the TBG binding activity by non-esterified fatty acids (NEFA) and the control of its synthesis by the thyroid status are also reported. Mono- and poly-unsaturated NEFAs are strong inhibitors of the TBG, although they affect TTR less readily. On the other hand, the biosynthesis and/or secretion of TBG, but not of TTR, is under thyroid-hormone control, experimental hypothyroidism inducing a marked increase of the serum TBG. The TBG of mouse behaves as a highly significant parameter of development, pointing to a likely important function of the protein in the process of maturation. Our finding of major TBGs in both euthyroid rats and mice suggests that TBG is more widely spread than was thought until now, but difficult to detect in certain species outside definite maturation stages.

INTRODUCTION

Thyroid hormones are essential for normal growth, but neither their precise actions nor the timing and mechanisms controlling these actions in the various metabolic processes of maturation are at present fully understood [1]. In the rat, the maturation of the hypothalamus–hypophysis–thyroid axis is a postnatal event [2], coinciding with characteristic developmental profiles of the circulating thyroid hormones [3] and of parameters related to their availability for targets, e.g. the affinities and concentrations of their brain receptors [4]. Recently, we found in this species a novel parameter of the thyroid function endowed with a striking ontogenic pattern. We showed [5–7] that the euthyroid rat possesses a previously unrecognized [8] thyroxine-binding globulin (TBG) analogous to the human TBG, and that this protein presents a postnatal transient increase which confers to the immature sera thyroxine (T_4)- and tri-iodothyronine (T_3)-binding activities 5–8 times higher than those of foetuses and adults.

Similarly to the rat, the euthyroid mouse was thought not to posses a TBG [8,9]. The transport of thyroid hormones was thought to be essentially effected by a prealbumin, i.e. the ubiquitous binding protein classically known as 'thyroxine-binding prealbumin', at present usually named 'transthyretin' (TTR). In the more recent electrophoretic studies [9] the mouse prealbumin appeared undistinguishable from albumin, and no T_4 binding was seen in the post-albumin region; however, T_4 binding to a post-albumin was evidenced in the sera from genetically obese *ob/ob* and *db/db* strains.

In a former study [10] we compared during the pre- and post-natal development of the mouse the serum abilities to bind T_4 and the corresponding immunoassayed concentrations of TTR. Similarly to our initial observation in the rat, which had led to the finding of a major rat TBG [5], we observed a high transient postnatal peak of T_4 binding contrasting with fairly unchanging TTR levels during the whole period of maturation. Electrophoretic studies revealed, in the sera with high binding activities, elevated T_4 binding to the post-albumin or 'TBG' region of the electrophoretograms.

Here we confirm and extend our finding of a hitherto unrecognized development-regulated TBG in the euthyroid mouse. We investigate its equilibrium binding parameters and structural microheterogeneity. We examine some of its regulatory properties, namely the effects on TBG binding activities of non-esterified fatty acids, acknowledged modulators of hormone–protein interactions, as well as the relation of TBG levels to the thyroid status. Development-related characteristics of mouse TTR are also presented for the first time.

EXPERIMENTAL

Animals, sera, proteins and hormones

Pooled sera of mice of different ages (mixed sexes up to 30 days) were from Charles River (strain CD1). Rat TTR and mouse α_1-foetoprotein (AFP) were purified as described [11,12] and used to raise rabbit antibodies for the immunoassay of mouse TTR

Abbreviations used: TBG, thyroxine (T_4)-binding globulin; T_3, tri-iodothyronine; TTR, transthyretin; i.e.f., isoelectric focusing; NEFA, non-esterified fatty acid; AFP, α_1-foetoprotein; PTU, propylthiouracil.

(which cross-reacts with the rat anti-TTR) and of mouse AFP. Anti-(rat albumin) serum (Nordic Immunology, Tilburg, The Netherlands) served for the quantification of mouse albumin. A highly enriched preparation of mouse TTR was prepared for binding studies using the technique for rat TTR purification [11], i.e. phenol precipitation and preparative electrophoresis, with an additional step of immunoaffinity chromatography on anti-(adult mouse serum) serum coupled to CNBr–Sepharose.

Hypothyroid male mice of age 60 days were obtained in the laboratory, by giving to 45-day-old animals propylthiouracil (PTU) (500 mg/litre) in their drinking water for 15 days. Replacement treatments were effected in parallel on a panel of age-matched mice, which received PTU under the same conditions together with a daily subcutaneous injection of T_3 (0.1 μg/g body wt.).

^{125}I-T_4 and ^{125}I-T_3, specific radioactivities > 1.2 mCi/mg, were from Amersham International. Radioinert T_4 and T_3 were from Sigma. Assay of serum T_4 concentrations was carried out with the Amerlex T_4 radioimmunoassay kit (Amersham International).

Equilibrium binding studies

Binding was measured with a batchwise gel-equilibration technique devised for steroid-binding studies [13]; conditions were as in the original technique, except for the replacement of the phosphate buffer by a Tris/HCl buffer (0.04 M, pH 7.4), containing NaCl (60 mM), KCl (4 mM), $CaCl_2$ (3 mM) and $MgCl_2$ (0.6 mM). Dialysis was for 1 h at 22 °C under agitation. The gel-equilibration technique and the graphic methods of Scatchard [14] and Rosenthal [15] were used to determine the combining-affinity indices or 'C values' ($l \cdot g^{-1}$), the association constants (K_a, M^{-1}) and the apparent maximum concentration of binding sites (nM). The C value measures the ability of a mixture of proteins to bind a small molecule and is given by the expression

$$C = (B/U)(1/P)$$

where B, U and P are the bound ligand, the unbound ligand and the concentration of proteins ($g \cdot l^{-1}$) respectively. Detailed descriptions and assessments of these techniques have been given elsewhere [16–18]. Protein concentrations were assayed as described by Lowry *et al.* [19], with rat serum albumin as standard.

Rocket electroimmunodiffusion

The technique of Laurell [20] was used to quantify the TTR, the albumin and the AFP in the sera under study.

PAGE studies

The electrophoretic behaviour of the thyroid-hormone-binding proteins was studied by cylindrical-gel or slab-gel PAGE (7% polyacrylamide) of sera pre-incubated overnight at 4 °C with tracer amounts of radioiodinated T_3 or T_4. In the cylindrical-gel PAGE experiments, before electrophoresis the labelled sera were stripped from possible excess of unbound radioiodinated hormones and endogenous free hormones by spun-down chromatography on microcolumns of Sephadex G-25 [21]. Electrophoresis was for 45 min at 80 V and 35 min at 160 V, at 4 °C, in a Tris (0.025 M)/glycine (0.2 M) buffer, pH 8.5. The labelled liganded proteins were located on the gels by counting successive 2 mm-thick transversal slices. Their migration was defined with respect to that of albumin revealed in parallel runs by Coomassie Brillant Blue staining.

In the slab PAGE experiments the electrophoretic runs were carried out in the same gel and buffer, without the spun-down-chromatography step. Electrophoresis was for 5 h under 140 V at 4 °C. The gel was dried without fixation and exposed for 5 h at −80 °C with a X-Omat S Kodak film.

Finally, a number of PAGE experiments were performed with a discontinuous buffer system (disc electrophoresis) essentially as described by Ornstein [22] and Davis [23], with the difference that the buffer for the separation gel was diluted by 1 in 2 to minimize ligand dissociation during runs.

Isoelectric-focusing (i.e.f.) studies

The labelling of T_4-binding proteins was studied after focusing of the sera (incubated with radioiodinated T_4) as described in [6]. LKB (Bromma, Sweden) Ampholine PAG plates pH 4–6.5 and pH 4–5 and the LKB Multiphor chamber and power supply were used. Sera (25 μl) were incubated for 1 h at room temperature with the hormone (100000 c.p.m.), and samples (5 μl) were applied at the cathodic side of plates. Runs were carried out, without prefocusing, at a maximum of 25 W at 10 °C, for 3 h. The pH gradient was assessed with an i.e.f. calibration kit (Pharmacia A.B., Uppsala, Sweden). Gels, dried for 4 h at 60 °C under vacuum, were exposed to Kodak X-Omat S films for 15 min–2 h at −80 °C with X-Omatic rapid screens.

I.e.f. experiments were also performed with the ^{125}I-T_4-labelled sera, subsequently incubated with neuraminidase (from *Vibrio cholera*; Behring Diagnostics, La Jolla, CA, U.S.A.). The proportion of components was 25 m units of enzyme: 25 μl of acetate buffer (0.2 M; pH 6.2): 25 μl of serum. Contact between enzyme and serum took place overnight at room temperature. These conditions were established as inducing maximal effects of the i.e.f. behaviour of TBG.

Inhibition studies with non-esterified fatty acids (NEFAs)

The effects of various NEFAs on the interactions of T_4 with the mouse serum proteins were studied in gel equilibration and PAGE experiments. The tested compounds were the stearic ($C_{18:0}$, double bond), oleic ($C_{18:1}$); arachidonic ($C_{20:4}$) and docosahexaenoic ($C_{22:6}$) acids. All were Sigma products.

In the gel-equilibration studies, solutions containing 1–4 μg of NEFA were evaporated in the test tube; the gel-equilibration milieu, the serum (0.8 μl) and the ^{125}I-T_4 (0.02 μCi) were added subsequently, and dialysis was performed as described above. In the PAGE studies, the NEFA solutions (5–20 μg) were evaporated and incubated overnight at 4 °C with 4 μl of serum and 0.1 μCi of ^{125}I-T_4, all in a final volume of 50 μl of Tris/glycine buffer, then submitted to PAGE on slab gels as described above.

RESULTS

Ontogeny of serum T_4- and T_3-binding activities as compared with the ontogeny of TTR, albumin and AFP concentrations

The binding activities measured at equilibrium with T_4 and T_3, and calculated as C values in whole sera from developing mice, are shown in Fig. 1. For the same sera the inset shows the immunoassayed concentrations of TTR and albumin, acknowledged to date as the only carriers of thyroid hormones in the euthyroid mouse, and of AFP, described as a low-affinity high-capacity thyroid-hormone binder in the rat [24]. These data confirm our former observation [10] of a high peak of T_4 binding between 3 and 5 days after birth and, further, show a similar ontogenetic pattern for the binding of T_3. In contrast with this trend, TTR keeps fairly constant levels of foetuses and postnatal mice up to 30 days, then significantly increases in the 60-day sera, which have the lowest thyroid-hormone-binding activities. As for albumin, it steadily increases, whereas AFP becomes hardly detectable 10 days after birth. Clearly, a strong binder of thyroid hormones, different from any of these three proteins, must be responsible for the high binding interactions of the perinatal ages.

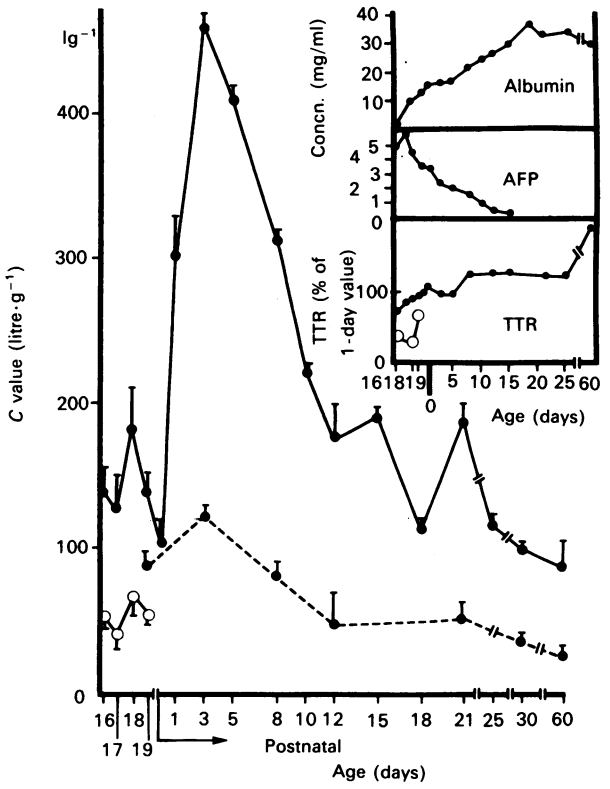

Fig. 1. Ontogenesis of T_4 and T_3 binding activities of whole sera, measured at equilibrium and calculated as *C* values

●——●, Binding of T_4; ●----●, binding of T_3; ○, binding of T_4 at different pregnancy ages. The inset shows ontogenesis of TTR, AFP and albumin concentrations in whole sera, measured by electroimmunodiffusion. TTR is calculated as a percentage of the value at 1 day.

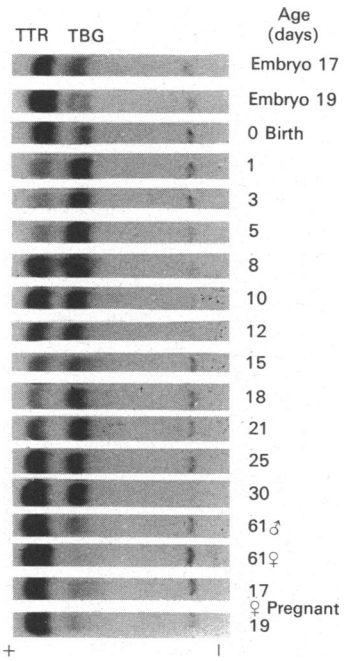

Fig. 2. Ontogenesis of TBG as against TTR labelling by ^{125}I-T_4 in slab PAGE experiments

Autoradiogram of an electropherogram obtained from sera pre-incubated with the hormone [25 μl of serum/0.5 μCi (28 μg) of T_4]. A 5 μl portion of each incubation solution was applied to the gel.

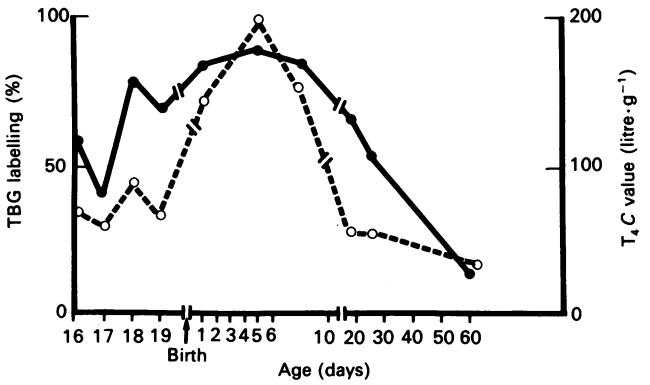

Fig. 3. Ontogenesis of T_4 binding activities of sera at equilibrium (*C* values) (○) compared with ontogenesis of labelling of TBG by ^{125}I-T_4 after cylindrical-gel PAGE [% of (TBG+TTR) labelling] (●)

In the PAGE experiments, 120 μg of serum proteins preincubated with 0.1 μCi of T_4 were applied to each gel.

Ontogeny of the serum T_4-binding proteins in PAGE experiments

Fig. 2 shows an autoradiograph of a slab-gel electropherogram obtained with ^{125}I-T_4-prelabelled sera from developing mice. It shows that, throughout development, the binding of the hormone is distributed between two main proteins: one is the acknowledged TTR in the albumin zone [9]; the other is a post-albumin, with the α_1-globulin migration characteristic for human and rat TBGs [5,8]. The relative labelling of TTR and TBG markedly shifts as a function of age. During postnatal development, at the ages at which the equilibrium binding indices of whole sera are at their highest, the bulk of bound radioactivity is on TBG. In contrast, in the adults, where the *C* values are lowest, the bulk of radioactivity is on TTR. This correlation between high binding and prevalence of TBG labelling is further illustrated in Fig. 3, which compares the developmental patterns of C-values measured by gel equilibration and of labelling of the TBG peak in cylindrical-gel PAGE experiments.

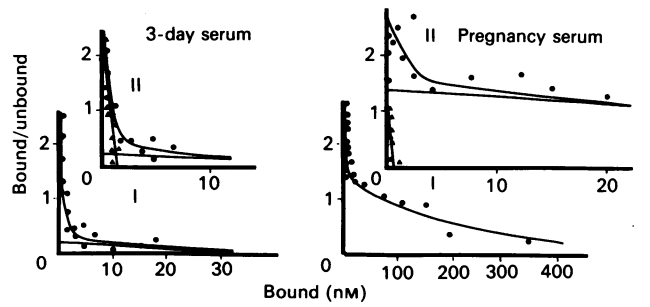

Fig. 4. Scatchard plots and Rosenthal corrections for the interactions of T_4 with pooled 3-day sera and pooled 19-day pregnancy sera

Reaction mixtures consisted of 200 mg of Sephadex G-25, 56 and 210 μg of serum proteins (for pups and pregnant females respectively), 0.08–7000 ng of T_4 in 2 ml of Tris/HCl buffer (0.04 M, pH 7.4). Dialysis was for 1 h at 22 °C with mild agitation. I, Whole sets of experimental data; II, enlargement of abscissae and Rosenthal correction for the highest-affinity class of sites (K_{a1}, n_1M_1). In the 3-day sera, $K_{a1} = 1.7 \times 10^9$ M^{-1} and $n_1M_1 = 37.5$ nmol/g of serum proteins. In the pregnancy sera, $K_{a1} = 1.66 \times 10^9$ M^{-1} and $n_1M_1 = 4.2$ nmol/g of serum proteins. Abcissae represent nmol of bound hormone/litre of external volume of the Sephadex suspension. Protein and bound hormone are excluded in the external volume, free hormone being distributed between internal and external volumes (cf. [13]).

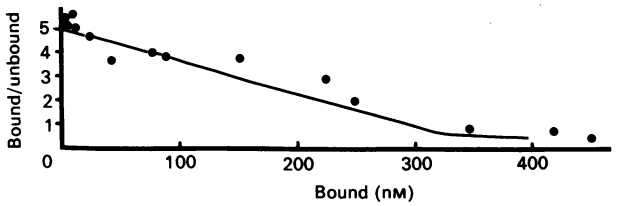

Fig. 5. Scatchard plot for the interaction of T_4 with highly enriched mouse TTR

Each reaction mixture contained 4.6 μg of protein. Conditions were as in Fig. 4. The K_a of the saturable site was 1.3×10^7 M^{-1}.

Since TTR concentrations vary but little during perinatal growth and even increase in adults, these results unambiguously demonstrate the perinatal increase of TBG and its responsibility for the high thyroid-hormone–serum interactions of this period.

Scatchard analysis of T_4 binding: studies with whole sera and with TTR

Fig. 4 allows comparison of Scatchard plots obtained with sera of maximal and minimal T_4-binding activities, i.e. from 3-day pups and 19-day pregnant females respectively. Rosenthal correction of the curves evidences in both sera a class of high-affinity sites ($K_{a1} = 1.7 \times 10^9$ M^{-1}), the maximum apparent concentrations of which are 8–9 times more elevated in pups than in mothers (37 as against 4 nmol/g of serum protein). As concentrations of TTR are similar in the two sera studied (cf. Fig. 1), it seems justifiable to infer that the $K_{a1} \sim 10^9$ M^{-1} sites are mostly on TBG, their increased levels accounting for the high T_4-binding activities of the 'immature' sera.

A second class of saturable sites, with a K_{a2} of about 10^7 M^{-1}, is present at comparable concentrations in the two sera. This fact, as well as the Scatchard analysis of a highly enriched TTR preparation (Fig. 5), which fails to evidence any quantifiable T_4 sites with higher than 10^7 M^{-1} association constants, strongly suggest that the $K_{a2} \sim 10^7$ M^{-1} serum binding sites for T_4 are essentially on TTR.

The purification of mouse TBG remains necessary for a complete analysis of the affinity and capacity equilibrium binding parameters of this protein.

I.e.f. studies

Studies from this and other laboratories demonstrated that human and rat TBGs are microheterogeneous proteins separable in four or five similar isoforms when isoelectrofocused in a pH gradient of 4–6.5 [6,25]. In Fig. 6 we show an autoradiogram obtained from mouse sera collected at different ages, prelabelled with ^{125}I-T_4 and submitted to i.e.f. in the 4–6.5 pH gradient. Reference rat and human sera are also presented. It may be seen that the TBGs of the three species display similar polymorphisms

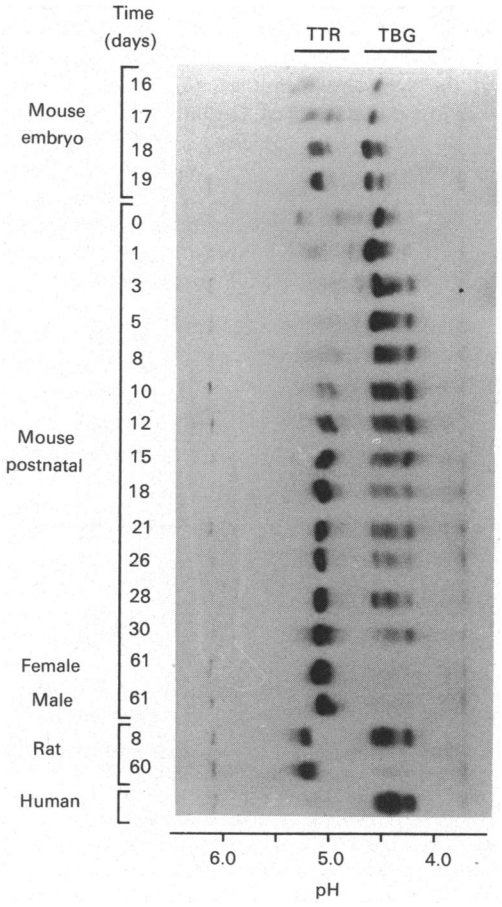

Fig. 6. Ontogenic study of mouse TBG and TTR in i.e.f. experiments

The Figure shows an autoradiogram of ^{125}I-T_4-labelled serum proteins after i.e.f. in the 4–6.5 pH gradient (see the Experimental section). Pre- and post-natal developing mouse, immature and adult rat, and adult human sera were examined.

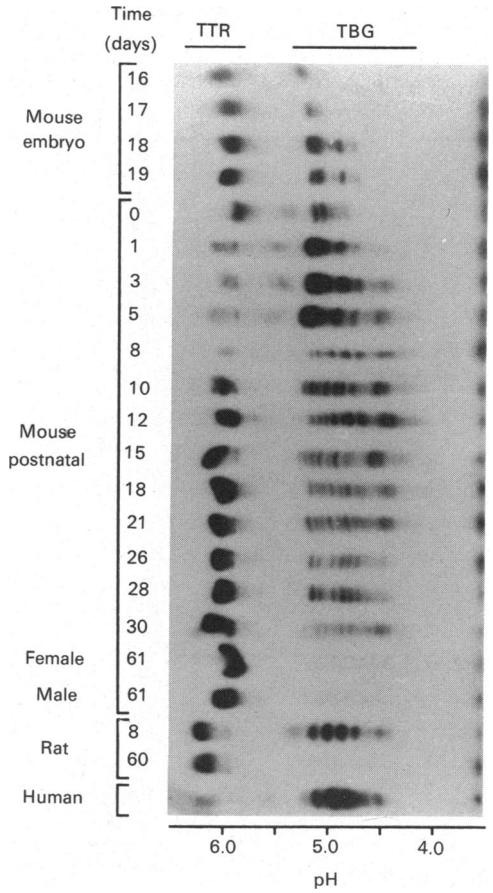

Fig. 7. Ontogenic study of mouse TBG and TTR in i.e.f. experiments.

The figure shows an autoradiogram of ^{125}I-T_4-labelled serum proteins after i.e.f. in the 4–5 pH gradient. Sera were as in Fig. 6.

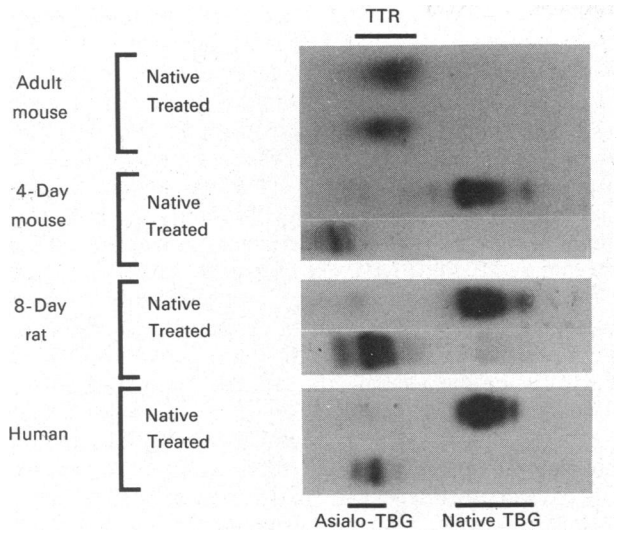

Fig. 8. Effect of neuraminidase treatment on TBG in i.e.f. experiments

The Figure shows an autoradiogram of ^{125}I-T_4-labelled mouse, rat and human serum proteins, treated with neuraminidase. I.e.f. was performed in the 4–6.5 pH gradient (see the Experimental section).

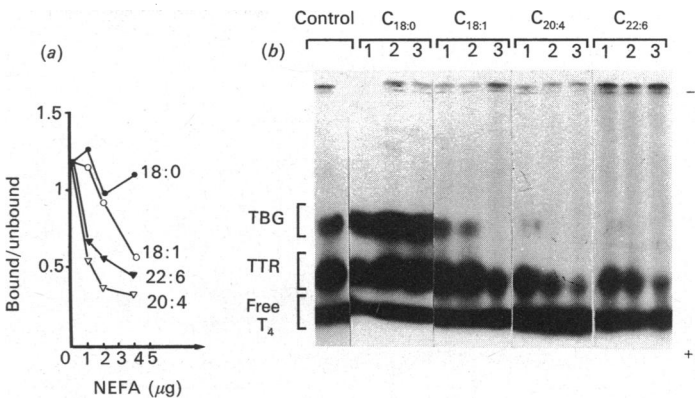

Fig. 9. Effects of NEFAs on T_4 interaction with 4-day mouse serum

(*a*) Gel equilibration: reaction mixtures consisted of 200 mg of Sephadex G-25, 28 μg of serum proteins, 0.02 μCi of ^{125}I-T_4, 0–4 μg of fatty acid, in 2 ml of Tris/HCl buffer (0.04 M, pH 7.4). (*b*) PAGE: autoradiogram of slab PAGE with 4-day mouse sera (140 μg of serum proteins) preincubated with 0.1 μCi of ^{125}I-T_4 in the absence (control) or presence of 5, 10 or 20 μg (designated 1, 2 and 3 respectively) of fatty acid. Disc electrophoresis was used.

consisting of four major isoforms with isolectric points (pI) at pH 4.3, 4.4, 4.5 and 4.6. As for the mouse TTR, it has a slightly more acidic pI (4.95) than that of rat or human TTRs (5.1), and at certain ages (10 or 12 days) it appears to be divided into two bands. The i.e.f. experiment evidences in the mouse sera the same age-dependent shifts of T_4 labelling between TBG and TTR as demonstrated by the PAGE studies in Figs. 2 and 3. It further shows that there are marked age-dependent variations in the relative abundance of the TBG isoforms, in particular the appearance only after birth of the more acidic bands. With ^{125}I-T_3 as ligand the same four major TBG isoforms have been evidenced (results not shown).

When i.e.f. is performed in the narrower gradient of pH 4–5 (Fig. 7) the microheterogeneity of mouse TBG is markedly increased, a further splitting of bands leading to the appearance of up to 12 isoforms. This phenomenon is not seen with the TBGs of the rat or the human reference sera.

To understand the possible role of the sialic acid moiety in the mouse TBG polymorphism, i.e.f. experiments have been carried out after neuraminidase treatment of mouse and of reference rat and human sera. The results (Fig. 8) show that, in the three species, desialylation, performed under identical conditions, induces two similar effects, i.e. the shift of the pI of TBG to higher pH and the decrease of the four major native bands to only two major bands. As discussed for human TBG [26] it may be deduced that the sialic acid is involved in the microheterogeneity of the murine TBGs. Additional structural factors, such as variations in amino acid composition, are probably responsible for the residual microheterogeneity persisting in the three species after the neuraminidase treatment.

Effects of NEFAs on the T_4–serum-protein interactions

NEFAs have been described as modulators of various hormone–protein interactions, in human as well as in rodent sera [17,27,28]. We have formerly shown in the rat that oleic acid inhibits T_4 and T_3 binding to immature and adult sera and that TBG is more sensitive than TTR to this inhibition [7]. Here we study the effects of four fatty acids, known to be present in significant amounts (micromolar concentrations) in the immature-rat sera [29], on the interactions of T_4 with the serum proteins of the immature mouse. The results are presented in Fig. 9. The gel-equilibration studies demonstrate dose-dependent and unsaturation-degree-dependent effects of the NEFAs. Whereas the saturated stearate ($C_{18:0}$) affects but little the T_4 binding to the whole sera, the unsaturated acids are strong inhibitors, the polyunsaturated docosahexaenoate ($C_{22:6}$) and arachidonate

Table 1. Effects of PTU-induced hypothyroidism on TBG and TTR: gel equilibration, cylindrical-gel PAGE and rocket electroimmunodiffusion (EID) studies

T_4 was measured by radioimmunoassay (r.i.a.). Controls are untreated 60-day-old males. PTU-treated mice and (PTU + T_3)-treated mice are 60-day-old males treated daily for 15 days, from the age of 45 days, with PTU and with PTU plus T_3 respectively. Values are means ± S.D. for duplicate experiments on five different sera. Statistical significance versus controls (Student's *t* test): ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, non-significant; ND, not determined.

Animals	*C* value (litre·g^{-1}) (gel equilibration)	10^{-3} × TBG labelling (c.p.m.) (PAGE)	TBG/TTR labelling ratio (PAGE)	[TTR] (% of control) (EID)	[T_4] (nmol/l) (r.i.a.)
Controls	39.5 ± 1.5	2.2 ± 0.35	0.26 ± 0.04	100 ± 6	93 ± 9.02
PTU-treated mice	63 ± 3.5***	5.4 ± 0.6	0.51 ± 0.05**	90 ± 6*	27 ± 1.8***
(PTU + T_3)-treated mice	41.5 ± 2.5NS	ND	ND	84 ± 13*	ND

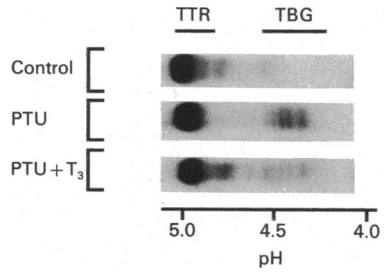

Fig. 10. Effect of PTU-induced hypothyroidism and of T_3 replacement on the T_4-binding proteins of 60-day mouse sera

The Figure shows an autoradiogram after i.e.f. of the ^{125}I-T_4-prelabelled serum proteins in the 4–6.5 pH gradient. The pH 4–5 zone of the autoradiogram is shown.

($C_{20:4}$) being more potent than the monounsaturated oleate ($C_{18:1}$). The PAGE studies, performed with NEFA/serum-protein ratios similar to those used in the equilibrium-binding studies, confirm these results and, moreover, allow discrimination between the responses of TBG and of TTR. Clearly, TBG is the more reactive target of the NEFA actions, the smaller doses of the inhibitors specifically preventing the binding of T_4 to this protein, whereas TTR is only inhibited when the higher amounts of NEFAs are tested. Whether the affinity or/and capacity binding parameters of the carriers are affected by the fatty acids remains to be determined in future studies.

Effects of PTU-induced hypothyroidism on TBG and on TTR

In adult rats hypothyroidism induced by thyroidectomy or PTU treatment leads to an important increase of the serum TBG [30]. Studies from this laboratory showed that PTU treatment of neonatal animals throughout development arrests the ontogenic fall of TBG while not affecting the TTR [18]. Here we examine the influence of hypothyroidism and thyroid-hormone replacement on the serum thyroid-hormone binders of adult mice. The results presented in Table 1 show that PTU treatment of 45-day-old mice, continued during 15 days, triggers highly significant increases of the C values for T_4, whereas replacement (simultaneous PTU and T_3 administration) maintains the T_4 binding indices at control level. Immunoassayed TTR levels are slightly decreased by either treatment, and TBG/TTR labelling ratios in cylindrical PAGE experiments are nearly doubled in PTU versus control rats.

The intensification of TBG labelling in 60-day-old PTU-treated mice, compared with age-matched untreated and (PTU + T_3)-treated mice, can be observed on the autoradiogram of the i.e.f. experiment in Fig. 10.

These data support the conclusion that the mouse, like the rat, responds to thyroid-hormone depletion by a significant increase of TBG, whereas its TTR appears not to be regulated by the thyroid status. The slight decrease in TTR observed in both PTU and replacement treatments (non-significant difference between these two groups) might represent a response to the stress of treatment.

DISCUSSION

We confirm the presence, in the euthyroid mouse, of a hitherto unrecognized thyroxine-binding globulin. We show that this protein presents a striking ontogenic pattern, with a high transient surge in the perinatal period, and that throughout development it plays crucial roles in the intensity of the serum interactions with T_4 or T_3. Indeed, the sera from pups aged 1–5 days display the highest thyroid-hormone-binding activities of ontogenesis, the highest concentrations of nanomolar K_d binding sites for T_4, and the strongest ^{125}I-T_4 labelling of the globulin in electrophoretic or i.e.f. experiments, together with comparatively low amounts of transthyretin (as measured by immunoassay), i.e. of the binding protein previously acknowledged as the sole specific thyroid-hormone carrier of the euthyroid mouse [8,9]. By contrast, the sera from 60-day-old adults contain the highest TTR levels of ontogenesis, together with low T_4 or T_3 binding activities, low concentrations of the nanomolar K_d sites and weak labelling of TBG in PAGE and i.e.f. experiments.

The TBG of the mouse appears as a significant parameter of development, similarly to that of the rat, thus reinforcing the notion of a likely important function of the protein in the process of maturation. Interestingly, the rat TBG becomes evident in the serum between 1 and 3 days after birth, whereas the mouse protein is already present in the foetus; indeed the whole ontogenic curve of TBG is shifted in the mouse towards earlier ages, with a postnatal peak at 3–5 days versus 6–8 days in the rat. Analogous kinetic differences between the two species have been observed for their oestrogen-binding AFPs and their corticosteroid-binding globulins, the ontogenic maxima of which occur at about 17 days in the foetal mouse and 19 days in the foetal rat [31,32]. Taken together, these results point to the involvement of the three high-affinity hormone binders, AFP, corticosteroid-binding globulin and TBG, in the multi-hormonal control of growth, at species-specific definite stages of development.

The regulatory properties of mouse TBG and TTR, either as concerns the modulation of their serum activities by NEFAs or the control of their synthesis by the thyroid status, are in many respects similar to those recently demonstrated in the rat [7–10] or to the available observations in man [27,33]. Among the evidenced analogies are the inhibition of TBG activity by oleic acid and the up-regulation of TBG in hypothyroid states (whether after PTU treatment or during physiological hypothyroidism of neonatal animals), contrasting with the non-response of TTR to hypothyroidism [7,34].

The lowest concentrations of unsaturated NEFAs which interfere with the binding of T_4 to mouse TBG are within the physiological concentrations of these compounds in the sera of the immature rodents [29]; the higher concentrations of NEFAs which inhibit both TBG and TTR are within the pathological limits observed, for example, in neonatal rats after adrenalectomy [29] or during turpentine-induced acute inflammation [35] and in man during severe non-thyroidal illnesses [36]. On the other hand, it is interesting to note that the inhibition of T_4 binding to mouse TBG is correlated not only with the amounts of the fatty acid inhibitors but also with their degree of unsaturation, similarly to the inhibition by fatty acids of the binding of oestrogens to the murine AFPs and of cortisol to human corticosteroid-binding globulin [27]. Changes in the composition of serum NEFAs in a number of pathophysiological conditions are well documented, e.g. the increased proportion of unsaturated NEFAs in rat foetuses and neonates [29] or in human non-thyroidal illnesses, including the acquired immunodeficiency syndrome [37]. Taken together, these data reinforce the notion that the levels and the nature of the serum NEFAs may play significant roles in modulating the bioavailability of the circulating thyroid as well as steroid hormones.

Furthermore, our studies evidence remarkable analogies between the TBGs of mouse, rat and man in respect of their thermodynamic binding parameters and their microheterogeneous structures.

Indeed, the present results are consistent with similar relations of affinity and capacity characteristics between TBG and TTR in the three species, i.e. nanomolar dissociation constants and low

capacities for the T_4 sites on TBG, in contrast with lower affinities (K_d 10^{-7}–10^{-8}) and higher capacities for the T_4 sites on TTR [5,7,8].

As to the microheterogeneities of the TBGs of mouse, rat and man, as evidenced by i.e.f., they are remarkably similar when a 4.5–6 pH gradient is used, and a comparable role of the sialic acid moiety in these polymorphisms is suggested by the similar i.e.f. behaviour of the TBGs of the three species after treatment with neuraminidase. In a narrower pH range (4–5) only the mouse TBG shows a splitting of bands, leading to increased numbers of isoforms. However, additional TBG bands have also been described in human sera from premature newborns [38], suggesting that, in man, the double-band appearance might be a characteristic associated with early development stages.

Taken together, the present studies show that the mouse, like the rat, is an adequate and accessible model for studying the regulatory and functional properties of human TBG. We think that particular biological significance ought perhaps to be sought in those characteristics which are common to the TBGs of the three species.

The genetic variants of the mouse appear as promising model systems to study the pre- and post-translational control of TBG and to clarify its roles. In the light of our present data, the appearance of a TBG-like protein in the sera of obese (*ob/ob*) and diabetic (*db/db*) mice [9] probably represents the increase of a TBG already existing in the lean controls, though at levels making detection comparatively difficult. These observations point to the usefulness of such variants, with specific metabolic defects, to evaluate the parameters of lipid or carbohydrate metabolism involved in TBG synthesis and activity. As, on the other hand, impaired transport of the thyroid hormones into the livers of the *ob/ob* mice has been evidenced [39], the use of such rich-in-TBG mutants to demonstrate directly a role of the protein in the compatmentation of thyroid hormones seems specially attractive.

Hypothyroid (*Hyt/Hyt*) mice have been shown to represent interesting models to study the effects of the thyroid hormones on the developmental changes of gene expression [40]. In view of our own data on the ontogeny of mouse TBG and on the thyroid-hormone-dependent production of the protein, the hypothyroid mutants may be envisaged as adequate experimental systems to clarify the relations between the thyroid-hormone action and TBG level at various stages of development.

In conclusion, the existence of major TBGs in the euthyroid rat and in the euthyroid mouse, two species which hitherto were thought to lack this protein, and the dramatic perinatal surges of both murine TBGs, suggest a possible ubiquitous presence of the protein, particularly associated with, and biologically important for, definite stages of maturation.

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