Identification by photoaffinity labelling of a pyridine nucleotide-dependent tri-iodothyronine-binding protein in the cytosol of cultured astroglial cells

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High-affinity 3,3',5-tri-iodo-L-thyronine (T_3) binding ($K_d \approx 0.3$ nM) to the cytosol of cultured rat astroglial cells was strongly activated in the presence of pyridine nucleotides. A 35 kDa pyridine nucleotide-dependent T_3 -binding polypeptide (35K-TBP) was photoaffinity labelled using underivatized [125 I] T_3 in the presence of pyridine nucleotides and the free-radical scavenger dithiothreitol. Maximum activations of T_3 binding and 35K-TBP photolabelling were obtained at approx. 1×10^{-7} M NADP⁺ or NADPH, or 1×10^{-4} M NADH. NAD⁺ and other nucleotides were without effect. NADPH is the form which activates T_3 binding and 35K-TBP photolabelling, since cytosol contains NADP⁺-reducing activity, and the activation of both processes in the presence of NADPH and NADP⁺ was prevented by an exogenous NADPH oxidation system. NADPH behaved as an allosteric activator of T_3 binding. The NADPH oxidation system

promoted the release of bound T_3 in the absence of any change in the total concentration of the hormone. The 35K-TBP photolabelling and [125 I] T_3 binding were similarly inhibited by non-radioactive T_3 (half-maximum effect at 0.5–1.0 nM T_3). The concentrations of iodothyronine analogues that inhibited both processes were correlated ($3,3',5$ -tri-iodo-D-thyronine $\geq T_3 > L$ -thyroxine $>$ tri-iodothyroacetic acid $> 3,3',5'$ -tri-iodo-L-thyronine). Molecular sieving and density-gradient centrifugation of cytosol identified a 65 kDa T_3 -binding entity, which included the 35K-TBP. These results indicate that 35K-TBP is the cytosolic entity involved in the pyridine nucleotide-dependent T_3 binding, and suggest that the sequestration and release of intracellular thyroid hormones are regulated by the redox state of astroglial cell compartment(s).

INTRODUCTION

Many small lipophilic molecules are bound to soluble (cytosolic) proteins within cells. These cytosolic proteins include the recently characterized non-receptor binding proteins for steroids [1,2], for retinoids [3] and for halogenated hydrocarbons [4], and the 'transfer' proteins for α -tocopherol [5] and phosphatidylinositol [6]. These proteins are believed to regulate the cytoplasmic availability of their ligands, mediate ligand distribution to intracellular targets, or participate in some of the ligand-induced biological effects.

Thyroid hormones [L-thyroxine (T_4) and 3,3',5-tri-iodo-L-thyronine (T_3)] are lipophilic iodoamino acids, sparingly soluble in aqueous media, which are bound *in vivo* to specialized proteins, both in the serum and in the cell. T_3 cytoplasmic binding sites have been known for many years (reviewed in [7]), but, because of their great number and their apparent low affinity, they were thought to constitute a passive intracellular reservoir of hormone.

Many of the biological effects of thyroid hormones are mediated through the binding of T_3 to nuclear receptors, which modify the transcription of specific genes (reviewed in [8]). However, several prenuclear steps appear to regulate the availability of T_3 to nuclear receptors. Specific, active processes are involved in the transport of thyroid hormones across the plasma membrane of a great number of cell types (reviewed in [9]). There have been reports of a concentration gradient of free T_3 between the cytosol and nuclei in several tissues in the rat [10], suggesting that there is an active-transport step at the nuclear envelope. The involvement of several cytosolic thyroid-hormone-binding proteins in the regulation of nuclear receptor occupancy has recently been documented. A human cytosolic T_3 -binding protein

(58 kDa) was shown to be a monomer of the tetrameric enzyme pyruvate kinase [11]. The interconversion between the T_3 -binding monomer and the non-binding tetramer was shown to regulate the transcriptional activity of T_3 nuclear receptors, perhaps by an effect on the steady-state concentration of free cytoplasmic T_3 [12]. Other cytosolic T_3 -binding proteins are under the control of pyridine nucleotides. Rat kidney cytosol contains a 58 kDa protein that binds T_3 with high affinity in the presence of NADPH or NADP⁺. The form activated by NADP⁺ was reported to bind to the nucleus *in vitro*, while that activated by NADPH does not [13], suggesting a role in the regulation of T_3 access to nuclear receptors.

The present work uses newborn-rat astroglial cells grown in primary culture to examine the T_3 -binding activity in the 100000 g supernatant of cell homogenates (cytosol) and to identify the T_3 -binding protein(s). Thyroid hormones play an essential role in the normal development of the mammalian central nervous system [14]. Newborn-rat astrocytes are target cells for thyroid hormones; they possess nuclear receptors and a 5'-deiodinase [15] which activates the prohormone, T_4 , to its intracellular active form, T_3 . The kinetics of the membrane thyroid-hormone transport system in rat cultured astroglial cells have been determined [16] and shown to be related to the neutral/aromatic amino acid transport system L₁ [17]. T_3 -binding sites have been found in the cytosol prepared from these cells [18], and the present study shows that these cytosolic high-affinity T_3 -binding sites are strongly dependent on the reduced form of pyridine nucleotides.

We have used photoaffinity labelling with underivatized [125 I] T_3 to identify the binding entity. This method of affinity labelling is regarded as highly specific [19] and has been used successfully to

Abbreviations used: T_3 , 3,3',5-tri-iodo-L-thyronine; D- T_3 , 3,3',5-tri-iodo-D-thyronine; r T_3 , 3,3',5'-tri-iodo-L-thyronine; T_4 , L-thyroxine; ip- T_2 , 3'-isopropyl-3,5-di-iodo-L-thyronine; triac, 3,3',5-tri-iodothyroacetic acid; DCC, dextran-coated charcoal; DTT, dithiothreitol; ED₅₀, median effective activating dose; 35K-TBP, 35 kDa pyridine nucleotide-dependent T_3 -binding polypeptide; IA, integrated absorbance.

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label T_3 receptors in the nuclei of liver cells [20] and a protein involved in T_3 transport across the erythrocyte plasma membrane [21]. The criteria of apparent affinity for T_3 , specificity of iodothyronine binding, and specificity of nucleotide activation were used to identify a soluble 35 kDa T_3 -binding polypeptide (35K-TBP), which is dependent on pyridine nucleotides, in rat astroglial cells. We show that the function of the 35K-TBP is regulated by the redox state of the cytosol.

MATERIALS AND METHODS

Chemicals

[3'- 125 I] T_3 (specific radioactivity 3 mCi/ μ g), X-ray films (Hyperfilm-MP), and intensifying screens (Hyperscreen) were purchased from Amersham-France (91944 Les Ulis, France). 3,3',5'-tri-iodo-L-thyronine (r T_3) from Calbiochem (France-Biochem, 92190 Meudon, France), 3'-isopropyl-3,5-di-iodo-L-thyronine (ip- T_3) from Henning (Berlin, Germany). Pyridine nucleotides, FAD, FMN and ATP were from Boehringer-Mannheim-France (38242 Meylan, France). Other nucleotides, T_3 , 3,3',5-tri-iodo-D-thyronine (D- T_3), T_4 , 3,3',5-tri-iodothyroacetic acid (triac), HCl-washed charcoal, Hepes buffer and BSA (Fraction V, ref. A 4503) were from Sigma-Chimie (38299 St.-Quentin-Fallavier, France). Dextran T-70, Sephacryl S-200HR and gel-filtration calibration kits were from Pharmacia-Biotech (78051 St.-Quentin-Yvelines, France). Other chemicals were purchased from Merck-Clévenot (94736 Nogent-sur-Marne, France).

Glial cell culture

Sprague-Dawley rats were from Iffa-Credo (69592 L'Arbresle, France). Glial cells from 2-day-old rat brain were cultured as previously described [17], in 60 cm² Petri dishes. The culture medium was replaced on day 7. Confluent cultures were used on days 12–15. The cells were identified as previously described [15].

Preparation of cytosol

The cell layer was rinsed twice with PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) and the cells were harvested in 0.5 ml of buffer A [1 mM MgCl₂, 0.32 M sucrose, 5% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 2 μ g/ml pepstatin, A, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 20 mM Hepes, pH 7.6] per dish. The cells were broken in a tight-fitting Dounce homogenizer and the homogenate was centrifuged at 105000 g for 1 h at 4 °C. The supernatant was treated with dextran-coated charcoal (DCC) [0.2% charcoal (w/v) and 0.1% dextran T-70 (w/v), final concentrations] in buffer B (0.32 M sucrose, 1 mM MgCl₂, 20 mM Hepes, pH 7.6) for 30 min, to remove endogenous T_3 . DCC was eliminated by centrifugation at 17000 g for 15 min, at 4 °C. The cytosol was kept frozen at –80 °C.

T_3 binding to cytosolic proteins

Aliquots of cytosol (6 μ g of protein) were incubated for 45 min at 0 °C, in a final volume of 0.3 ml of buffer C [buffer B plus 1 mM dithiothreitol (DTT)] containing [125 I] T_3 (1×10^{-10} M; 0.2 μ Ci/ml) and appropriate nucleotides and/or additives, depending on the experiment. The non-saturable bound radioactivity was determined in the presence of 1×10^{-6} M unlabelled T_3 . Bound and free hormone fractions were separated by DCC treatment (same concentrations as above). The samples were shaken for 1 min at 4 °C and centrifuged (10000 g, 3 min). Dissociation of bound T_3 during DCC treatment was approx. 10% (as estimated from the time of half-dissociation, see Figure

Figure 1 [125 I] T_3 binding to astroglial cell cytosol

Cytosol was incubated at 0 °C with [125 I] T_3 in the presence or absence of pyridine nucleotides. Free and bound [125 I] T_3 were separated by DCC treatment. (a) Time course of [125 I] T_3 binding in the absence (○) or presence of 2×10^{-9} M (▲) or 3×10^{-7} M (●) NADPH. (b) Effect of increasing concentrations of NADPH (●), NADP⁺ (■), NADH (▲) and NAD⁺ (○) on [125 I] T_3 binding measured after a 45-min incubation. The non-saturable binding index was measured in the presence of a 10^4 -fold excess of unlabelled T_3 and was subtracted from the total binding index, calculated as described in the Materials and methods section.

5). Bound [125 I] T_3 was determined in duplicate aliquots of supernatant. The radioactivity was measured in a γ -counter.

Photoaffinity labelling

Aliquots of cytosol (26 μ g of protein) were incubated for 30 min at 0 °C in 0.4 ml of buffer B or C, containing 2×10^{-10} M [125 I] T_3 (0.4 μ Ci/ml), in the presence or absence of various pyridine nucleotides and/or unlabelled thyroid-hormone analogues. The samples were transferred to 3-cm² glass cups placed on ice, and exposed to u.v. light (150 W mercury arc tube; Hanau TQ 150, Heraeus-France, 91941 Les Ulis, France) at a distance of 4.5 cm from the tube axis. A water-cooled Pyrex jacket prevented the transmission of short-wave u.v. (wavelength < 300 nm), so that predominant emission wavelengths in the u.v. were 313 and 366 nm. The samples were routinely irradiated for 20 min.

SDS/PAGE and two-dimensional electrophoresis

The proteins in the irradiated samples were trichloroacetic acid-precipitated [22]; the pellets were then dissolved in electrophoresis

sample buffer [2% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (w/v) glycerol, 60 mM Tris/HCl, pH 6.8] and neutralized with 1 M NaOH. The samples were boiled for 5 min and electrophoresed in SDS/10% (w/v) polyacrylamide slab gels (1.5 mm thick) by the method of Laemmli [23], with a 4% (w/v) polyacrylamide stacking gel. The molecular masses of the ^{125}I -labelled peaks were estimated using molecular-mass standards (14.4 to 94 kDa). Gels were fixed, stained (Coomassie Blue R250), destained and dried. The bands of interest were cut out and the radioactivity was measured in a γ -counter, or the gels were exposed to X-ray film with an intensifying screen at -80°C . The autoradiographs were scanned with a densitometer (BioImage Electrophoresis System, Millipore Corp., Bedford, MA, U.S.A.) which calculated the integrated absorbance (IA) of each band.

The cytosol was applied to a 4% acrylamide gel containing 9.2 M urea, for isoelectric focusing [24], followed by 10% polyacrylamide/SDS gel electrophoresis, as described above. The ^{125}I T₃-labelled protein was detected by autoradiography of the gel.

Gel filtration

Astroglial cell cytosol (1.1 mg of protein in 2 ml) was applied to a Sephacryl S-200HR column (70 cm $\times$ 1.6 cm) equilibrated with buffer C, and the proteins were eluted with the same buffer. The column was calibrated using gel-filtration calibration kits (human immunoglobulin, BSA, ovalbumin and chymotrypsinogen A, 1 mg each in 1 ml). The void volume (V_0) was determined with Dextran Blue. A plot of $\{-\log [(V_e - V_0)/(V_t - V_0)]\}^{1/2}$ against R_s was constructed, where V_e is the elution volume of the calibration proteins, R_s is the corresponding Stokes radius, and V_t is the total volume of the column. The R_s of the T₃-binding protein was determined from this linear plot, knowing its V_e .

Sucrose-density-gradient centrifugation

Cytosol was diluted 1:3 in buffer D (1 mM MgCl₂, 1 mM DTT, 20 mM Hepes, pH 7.6) in order to lower the sucrose concentration below 5% and NADPH was added to a final concentration of 1×10^{-7} M. Aliquots (0.3 ml, 70 μg of protein) were layered on to 5–20% sucrose density gradients (4.7 ml) made in buffer D containing 1×10^{-7} M NADPH. The gradients were centrifuged at 45000 rev./min for 18 h at 2°C (rotor SW 50.1, Beckman) and fractions collected. The gradients were calibrated with glucose oxidase (7.9 S), BSA (4.3 S) and ovalbumin (3.6 S). The S values were determined by three experiments.

Miscellaneous methods

The NADPH or NADH fluorescence was measured at $0-3^\circ\text{C}$ in a Hitachi F-2000 fluorimeter equipped with a thermostatically controlled cuvette. The excitation and emission wavelengths were 343 and 440 nm respectively. The protein concentration was measured by the method of Bradford [25] using BSA as the standard.

Expression of results

Since the amount of bound T₃ at equilibrium may be high (30–60%) relative to the total amount of T₃ (1×10^{-10} M) used in the assays, a 'binding index' was defined as the ratio of bound to unbound T₃ concentrations. This ratio varied linearly with the amount of cytosolic protein up to a level of 9 μg /assay. The

results are expressed as mean $\pm$ S.D. The values of the median effective activating doses (ED_{50}) of nucleotides were determined by linear regression using a logit-log plot of the data. The values of the equilibrium dissociation constant (K_d), of the inhibition constant (K_i) and of the maximum number of binding sites (B_{max}) were obtained by non-linear least-square curve fitting (mean $\pm$ S.E.M. of estimate), using an adaptation (Biosoft, Cambridge CB2 1JP, U.K.) for the Apple-Macintosh of the LIGAND program of Munson and Rodbard [26].

RESULTS

Nucleotide dependence of T₃ binding to astroglial cytosolic proteins

The time course of [^{125}I]T₃ binding to cytosol in the presence of 3×10^{-7} M NADPH, at 0°C , is shown in Figure 1(a). Equilibrium binding was reached after 45 min of incubation. In the presence of 2×10^{-9} M, equilibrium binding was reduced by half, and was almost suppressed in the absence of nucleotide.

Addition of an exogenous NADPH-regenerating system (1×10^{-5} M glucose 6-phosphate and 0.09 unit/ml glucose-6-phosphate dehydrogenase) to the T₃-binding assay did not enhance the activation of T₃ binding promoted by either NADPH concentration (results not shown). Under these conditions, the conversion of 3×10^{-7} M NADP⁺ into NADPH was complete in 10 min at $0-3^\circ\text{C}$, as monitored by NADPH fluorescence (see the Materials and methods section).

The T₃-binding index (ratio of bound to unbound T₃, as defined in the Materials and methods section) was measured after a 45-min incubation. In the absence of nucleotides, it was 0.08 ± 0.04 ($n = 10$). The non-saturable binding was $50 \pm 10\%$ of the total binding. The saturable [^{125}I]T₃ binding index was increased in a dose-dependent manner when NADP⁺ or NADPH (1×10^{-10} to 3×10^{-7} M) was added to the incubation medium (Figure 1b). The dose-response curves of activation in the

Figure 2 Autoradiographs of SDS/polyacrylamide gels of cytosolic proteins after photoaffinity labelling with [^{125}I]T₃.

Cytosol was incubated for 30 min at 0°C with [^{125}I]T₃, in the absence (a) or presence (b) of 1 mM DTT, without nucleotide (lanes 1) or with 1×10^{-7} M NADPH (lanes 2), 1×10^{-7} M NADP⁺ (lanes 3), 1×10^{-4} M NAD⁺ (lanes 4), or 1×10^{-4} M NADH (lanes 5). The samples were placed on ice and u.v.-irradiated for 20 min. SDS/PAGE and autoradiography were performed as described in the Materials and methods section. A Coomassie-blue-stained lane is shown in (c) and molecular masses of size markers are shown on the right.

Figure 3 Time course and dose-dependent stimulation by NADPH of 35 kDa polypeptide photolabelling

Cytosol was incubated (30 min, 0 °C) with [125 I] T_3 in the presence of 1 mM DTT and NADPH or NADP⁺. The samples were u.v.-irradiated and subjected to SDS/PAGE and autoradiography. The integrated absorbance (IA) of the bands corresponding of the 35 kDa polypeptide was determined as described in the Materials and methods section. (a) Incubation was performed with 1×10^{-7} M NADPH in the absence (●) or presence (○) of 1×10^{-6} M unlabelled T_3 , and the samples were exposed to u.v. light for increasing times. (b) Incubation was performed in the presence of increasing concentrations of NADPH (●) or NADP⁺ (△) and samples were exposed to u.v. light for 20 min. IA values obtained in the experiment with NADP⁺ were divided by 1.66, in order to have the same arbitrary IA value at 1×10^{-7} M of each nucleotide.

presence of NADP⁺ and NADPH were very similar: the ED₅₀ was approx 2×10^{-9} M in both cases. The maximum binding index, measured using different cytosol preparations, was obtained in the presence of approx. 1×10^{-7} M each nucleotide. The value was 0.5 ± 0.2 in the presence of NADP⁺ ($n = 7$), as well as in the presence of NADPH ($n = 9$), corresponding to an approx. 12-fold stimulation. Non-saturable binding was not increased by the nucleotides.

The ED₅₀ of NADH was approx. 7×10^{-6} M (Figure 1b). The maximum binding index, measured at 1×10^{-4} M NADH in three experiments performed with different cytosols, was 0.4 ± 0.1 , corresponding to an approx. 10-fold stimulation. This maximum binding index was not statistically different from that obtained in experiments performed with NADPH ($P > 0.2$). While NAD⁺ (up to 1×10^{-4} M) did not activate T_3 binding (Figure 1b), it did not block the activating effect of 1×10^{-7} M NADP⁺, 1×10^{-7} M NADPH, or 1×10^{-4} M NADH (results not shown). No additional activation was observed when NADP⁺ and NADPH (1×10^{-7} M each), or NADH (1×10^{-4} M) and NADPH (1×10^{-7} M) were present together (results not shown). FAD, FMN, and oxidized and reduced NMN (up to 1×10^{-3} M) had no effect on the saturable T_3 binding. ATP (1×10^{-3} M) increased the binding index 1.3–2.6 times (range of two experiments), while GTP (1×10^{-3} M) had no effect.

Removing DTT from the incubation medium did not significantly alter the maximum binding index measured in the presence of 1×10^{-7} M NADP⁺ ($n = 4$, $P > 0.4$) or NADPH ($n = 4$, $P > 0.9$), or 1×10^{-4} M NADH ($n = 3$, $P > 0.2$).

Identification of the nucleotide-activated T_3 -binding proteins

Cytosol was incubated with [125 I] T_3 in the presence or absence of various pyridine nucleotides until binding equilibrium was reached. [125 I] T_3 was then cross-linked to its binding proteins by u.v.-irradiation (photolabelling). Figure 2 shows the auto-

Figure 4 Effect of astroglial cell cytosol on the redox state of NADPH and NADP⁺

Cytosol (20 µg of protein/ml) was incubated in buffer C, at 0–3 °C, with 3×10^{-7} M NADPH (●) or NADP⁺ (○). The fluorescence of NADPH was monitored as a function of time. The results are expressed as percentage of the fluorescence measured at time 0 in the assay containing 3×10^{-7} M NADPH.

radiographs obtained after PAGE of samples photolabelled in the presence or absence of DTT.

Several photolabelled polypeptides were detected in the absence of DTT and nucleotide. The photolabelling of only a 35 kDa polypeptide was increased approx. 10 times in the presence of 1×10^{-7} M NADP⁺, 1×10^{-7} M NADPH, or 1×10^{-4} M NADH (Figure 2a), and was abolished by 1×10^{-6} M non-radioactive T_3 (result not shown). In contrast, 1×10^{-4} M NAD⁺ had no effect on the photolabelling of any polypeptide (Figure 2a). In the presence of DTT, the photolabelling of the 35 kDa polypeptide was still enhanced approx. 9-fold by NADP⁺, NADPH and NADH, but not by NAD⁺ (Figure 2b) and was abolished by 1×10^{-6} M unlabelled T_3 (result not shown). The photolabelling of all other polypeptides was suppressed by DTT.

The time course of photolabelling of the 35 kDa polypeptide in the presence of 1×10^{-7} M NADPH and DTT (Figure 3a) showed that the intensity of labelling increased with the time of u.v.-irradiation, and was near-maximum between 20 and 40 min. No other photolabelled polypeptide was detected on the autoradiographs whatever the irradiation time. An irradiation time of 20 min was routinely used. The NADPH-activated photolabelling of the 35 kDa polypeptide was abolished by 1×10^{-7} M non-radioactive T_3 .

The photolabelling of the 35 kDa polypeptide was gradually increased in the presence of concentrations of NADP⁺ or NADPH increasing up to 1×10^{-7} M (Figure 3b). The half-maximum activation was obtained at approx. 1×10^{-8} M of each nucleotide.

Effect of the NADPH/NADP⁺ redox state on T_3 -binding activation and 35 kDa polypeptide photolabelling

Cytosol (same protein concentration as that used in the binding assays) was incubated with NADPH or NADP⁺ (3×10^{-7} M) at 0–3 °C, and NADPH fluorescence was monitored as a function of time. Figure 4 shows that NADP⁺ was strongly reduced to NADPH, whereas NADPH was only weakly oxidized. After incubation of cytosol with 1×10^{-4} M NAD⁺ for 45 min, only a

Table 1 Effect of NADPH oxidation system on T_3 binding and 35 kDa polypeptide photolabelling

Cytosol (20 μ g of protein/ml) was incubated at 0 °C in the presence of 3×10^{-7} M NADP⁺ or NADPH, with or without 1×10^{-4} M GSSG plus 0.33 unit/ml glutathione reductase (GR) (NADPH oxidation system). The fluorescence of NADPH was measured as described in the Materials and methods section and expressed as the percentage of the fluorescence measured at time 0, in the assay containing 3×10^{-7} M NADPH. T_3 binding was determined as in Figure 1 and 35 kDa polypeptide photolabelling was determined as in Figure 3. Values are expressed as the percentage of T_3 binding or photolabelling measured in the absence of the NADPH oxidation system.

Nucleotide present at time 0...	NADPH present after 45 min (%)		$[^{125}\text{I}]\text{T}_3$ binding (%)		Photolabelling (%)	
	NADP ⁺	NADPH	NADP ⁺	NADPH	NADP ⁺	NADPH
Without GSSG + GR	54–73*	90–97*	100	100	100	100
With GSSG + GR	0	0	15–20*	10–10*	3	1

* Range of two experiments.

Figure 5 Dissociation of T_3 binding

Cytosol was incubated at 0 °C with $[^{125}\text{I}]\text{T}_3$ up to equilibrium binding (45 min) in the presence of 1×10^{-7} M NADPH. Dissociation, at 0 °C, was initiated (time 0) by adding either DCC suspension (○) or an exogenous NADPH-oxidation system (1×10^{-4} M GSSG + 0.33 unit/ml glutathione reductase) (●). Under the first condition, dissociation was stopped, at the times indicated, by centrifugation of the DCC-containing assays. Under the second condition, dissociation was stopped by a 1-min DCC treatment. Bound $[^{125}\text{I}]\text{T}_3$ was measured in the supernatants. The results are given as the percentage of T_3 bound at time 0 of dissociation. The exponential regression curves are given ($r = 0.99$ in both conditions).

0.2% conversion of NAD⁺ into NADH (approx. 2×10^{-7} M) was measured fluorimetrically (results not shown).

Full activations of T_3 binding and photolabelling were measured after a 45-min incubation at 0–3 °C in the presence of 3×10^{-7} M NADPH or NADP⁺. Table 1 shows that, under these conditions, more than half of the NADP⁺ was reduced, whereas little NADPH was oxidized. One min after adding exogenous NADPH oxidation system (GSSG plus glutathione reductase) to the assays containing NADPH or NADP⁺, the NADPH fluorescence fell below the detection limit of the method ($\leq 1 \times 10^{-8}$ M NADPH), and remained at this level for at least 60 min. Under these conditions, T_3 binding was strongly reduced and the 35 kDa polypeptide photolabelling was almost suppressed, when measured in the presence of NADPH or NADP⁺ (Table 1).

Figure 6 Specificity and saturability of $[^{125}\text{I}]\text{T}_3$ binding to cytosolic proteins

Cytosol was incubated with $[^{125}\text{I}]\text{T}_3$ and with increasing concentrations of unlabelled D- T_3 (○), T_3 (●), T_4 (■), triac (□), ip- T_2 (△) or r- T_3 (▲), in the presence of 1×10^{-7} M NADPH (a), or with increasing concentrations (1×10^{-10} to 1×10^{-7} M) of unlabelled T_3 , in the presence of 1×10^{-9} M (○) or 1×10^{-7} M (●) NADPH (b). Bound $[^{125}\text{I}]\text{T}_3$ was determined as described in the Materials and methods section. (a) The values are expressed as percentage of the binding index determined in the absence of unlabelled analogue, and plotted versus the analogue concentrations. (b) Binding data were plotted according to the Scatchard representation. The best fits of the data were calculated with the program LIGAND.

The reversibility of T_3 binding and that of NADPH activation are illustrated in Figure 5. After T_3 binding reached equilibrium, dissociation at 0 °C was initiated by addition of either DCC or

Figure 7 Specificity and saturability of [¹²⁵I]T₃ photolabelling of the 35 kDa polypeptide

Cytosol was incubated with [¹²⁵I]T₃ and increasing concentrations of thyroid hormone analogues or unlabelled T₃, in the presence of 1×10^{-7} M NADPH. The samples were u.v.-irradiated, and subjected to SDS/PAGE and autoradiography. (a) The 35 kDa area region of the autoradiographs is shown as a function of thyroid-hormone analogue concentrations. (b) The radioactivity of the gel bands corresponding to the labelled 35 kDa polypeptide was determined in a γ -counter. The results are expressed as percentages of control (band radioactivity measured in absence of unlabelled T₃).

the exogenous NADPH oxidation system. In both cases, dissociation followed first-order kinetics, as calculated by exponential regression ($r = 0.99$). Half-time of T₃ dissociation was approx. 6 min under the first condition, and approx. 30 min under the second one.

Specificity and saturability of T₃ binding and photolabelling

Figure 6(a) shows the inhibition of the NADPH-activated [¹²⁵I]T₃-binding by increasing concentrations of thyroid hormone analogues. The rank order of inhibition was D-T₃ > T₃ > T₄ > triac > ip-T₂ > rT₃. The values of K_i (nM) calculated with the program LIGAND were: D-T₃, 0.19 ± 0.02 ; T₃, 0.38 ± 0.03 ; T₄, 1.5 ± 0.1 ; triac, 4.5 ± 0.9 ; ip-T₂, 27 ± 6 ; and rT₃, 61 ± 7 .

A Scatchard plot of T₃ (1×10^{-10} M to 1×10^{-7} M) binding data in the presence of 1×10^{-7} M NADPH shows that only one class of T₃-binding sites (Figure 6b) was detected. Decreasing the

Figure 8 Sephacryl S-200 column chromatography and sucrose-density-gradient centrifugation of cytosolic T₃-binding activity

(a) Cytosol was applied to a Sephacryl S-200 HR column equilibrated with buffer C, and eluted with the same buffer. [¹²⁵I]T₃ binding index was measured in the absence (○) or presence (●) of 1×10^{-7} M NADPH. The protein concentration was measured (△). The elution positions of the column void volume (A), human immunoglobulin (B), BSA (C), ovalbumin (D) and chymotrypsinogen A (E) are indicated at the top of the figure. Inset, Fractions 44, 45, 47, 49 and 50 (80 μ l aliquots) were incubated in the presence of [¹²⁵I]T₃ and 1×10^{-7} M NADPH, u.v.-irradiated and subjected to SDS/PAGE and autoradiography. The integrated absorbance (IA) of the bands corresponding to the 35 kDa polypeptide was determined as described in the Materials and methods section. Columns A, B and C show the IA of fraction 47 incubated in the absence (A) or presence of 1×10^{-7} M NADPH (B), or in the presence of 1×10^{-7} M NADPH plus 1×10^{-6} M unlabelled T₃ (C). (b) Cytosol was centrifuged through a 5–20% sucrose density gradient containing 1×10^{-7} M NADPH. The [¹²⁵I]T₃ binding was measured in the presence of 2×10^{-7} M NADPH. The migration of reference markers is shown.

concentration of NADPH to 1×10^{-9} M resulted in an increase of K_d , while the B_{max} value was unchanged. In the absence of nucleotide, binding was too weak to allow precise determination of K_d and B_{max} . The binding data of 5 experiments performed with different cytosols at 10^{-7} M NADPH were analysed using the program LIGAND. The K_d was 0.31 ± 0.04 nM and the B_{max} was 10.0 ± 1.6 pmol/mg of protein or 38 ± 6 pmol/mg of DNA.

Figure 7(a) shows that the intensity of photolabelling of the 35 kDa polypeptide decreased as a function of the concentration of the various thyroid hormone analogues. The rank order of inhibition was D-T₃ $\approx$ T₃ > T₄ > triac > rT₃.

Non-radioactive T₃ inhibited the NADPH-activated photolabelling of the 35 kDa polypeptide in a dose-dependent manner (Figure 7b). The half-maximum inhibition was obtained at 5×10^{-10} – 1×10^{-9} M non-radioactive T₃, and inhibition was almost complete at 1×10^{-8} M. Similar results were obtained in another photolabelling experiment.

Figure 9 Two-dimensional gel electrophoresis of [^{125}I] T_3 photolabelled cytosol

Cytosol was incubated at 0 °C with [^{125}I] T_3 in the presence of 1×10^{-7} M NADPH, and u.v.-irradiated. The radiolabelled proteins were analysed by two-dimensional gel electrophoresis and autoradiography. Only the 25–45 kDa region of the Coomassie-Blue-stained gel (a), with molecular masses of size markers, and of the corresponding autoradiograph (b) are shown.

Physicochemical properties of the nucleotide-dependent [^{125}I] T_3 -binding protein

Chromatography of astroglial cytosol on Sephacryl S-200HR (Figure 8a) revealed a single peak of [^{125}I] T_3 binding activated in the presence of NADPH. The Stokes radius (R_s) of the T_3 -binding protein, determined with reference to the calibrating proteins, was 31 ± 2 Å (three experiments performed with different cytosols). The photolabelling of peak fractions by [^{125}I] T_3 in the presence of 1×10^{-7} M NADPH, revealed the presence of a single photolabelled polypeptide with a molecular mass of 35 kDa, whose photolabelling was reduced approx. 30-fold in the absence of NADPH and abolished by 1×10^{-6} M non-radioactive T_3 (Figure 8a, inset). Centrifugation of the cytosol through a 5–20% sucrose density gradient determines a sedimentation coefficient of 4.9 ± 0.2 S ($n = 3$) for the T_3 -binding activity, measured in the presence of NADPH (Figure 8b). The apparent molecular mass of the binding protein, calculated from the R_s and the sedimentation coefficient was approx. 65 kDa.

Cytosol was photolabelled and analysed by two-dimensional gel electrophoresis. A single labelled spot was detected by autoradiography, migrating as a 35 kDa polypeptide having a pI of approx. 5 (Figure 9). No detectable Coomassie-Blue-stained protein corresponded to the labelled spot.

DISCUSSION

Photoaffinity labelling with underivatized T_3 identified a 35 kDa T_3 -binding polypeptide (35K-TBP) which is involved in the high-affinity T_3 binding to cytosolic proteins of astroglial cells. The mechanism for covalent labelling may imply phenyl radicals generated by u.v.-irradiation of T_3 , and aromatic residues located in the T_3 -binding site [19]. U.v.-irradiation caused the labelling of

a single polypeptide, in the presence of [^{125}I] T_3 , NADP⁺ or NADPH, and DTT. The complete inhibition of photolabelling of this 35 kDa polypeptide by an excess of non-radioactive T_3 indicates that saturable T_3 -binding sites were photolabelled. The soluble extract was prepared in the presence of a mixture of protease inhibitors, so that it is unlikely that the 35K-TBP was a proteolytic product of a larger species. In addition, the absence of any other photolabelled polypeptide during the entire time course of u.v.-irradiation indicates that no u.v.-induced peptide cleavage or cross-linking occurred.

Several polypeptides were photolabelled by [^{125}I] T_3 in addition to 35K-TBP, in the absence of DTT. However, their photolabelling was not stimulated by pyridine nucleotides, indicating that these polypeptides are not involved in nucleotide-activated T_3 binding. In addition, their photolabelling was not inhibited by excess non-radioactive T_3 , consistent with non-saturable T_3 binding, and/or non-specific photolabelling. DTT was reported to be a powerful scavenger of free radicals generated by u.v.-irradiation [20,27]. Its presence during u.v.-irradiation considerably decreased the covalent attachment of T_3 to low-specificity or low-affinity sites [21]. DTT did not prevent the photolabelling of 35K-TBP, indicating that there is a tight interaction between T_3 and the 35K-TBP.

The characteristics of 35K-TBP photolabelling correlate well with those of T_3 binding to astroglial soluble protein(s). The intensity of photolabelling and T_3 binding to cytosol were maximally stimulated in the presence of the same concentrations of NADP⁺, or of NADPH, or of NADH. Omission of DTT had no effect on the maximal activation of either T_3 binding or 35K-TBP photolabelling by pyridine nucleotides. T_3 binding and 35K-TBP photolabelling were half-activated in the presence of similar NADPH concentrations and of similar NADP⁺ concentrations. In addition, NADPH and NADP⁺ appeared to be equally potent. Furthermore, NAD⁺ activated neither 35K-TBP photolabelling nor T_3 binding.

The effects of NADPH and NADP⁺ on T_3 binding were not additive, indicating that the same T_3 -binding sites were activated by both nucleotides. The fact that NADPH and NADP⁺ have identical ED_{50} values suggested that the activation process involved enzymic reduction of NADP⁺ or/and oxidation of NADPH. In fact, a large part of the NADP⁺ was readily reduced, even at 0 °C, by an endogenous NADP⁺-reducing activity present in the cytosol. Although an endogenous NAD⁺-reducing activity was present in the cytosol, the concentration of NADH produced during the incubation was too low (approx. 2×10^{-7} M) to activate T_3 binding.

The reduction of NADP⁺ by cytosol component(s) was reversed, and NADPH was oxidized, in the presence of an exogenous NADPH oxidation system (GSSG + glutathione reductase). There was very much less activation of T_3 binding by NADPH or NADP⁺ under these conditions. This indicates that the activation by NADP⁺ is indirect, and is due to its prior reduction to NADPH. Similarly, in the presence of the exogenous NADPH oxidation system, NADPH and NADP⁺ no longer activated 35K-TBP photolabelling.

[^{125}I] T_3 binding and 35K-TBP photolabelling were half-inhibited by similar unlabelled T_3 concentrations. In addition, the orders of potency of unlabelled thyroid hormone analogues, for inhibiting [^{125}I] T_3 binding and 35K-TBP photolabelling, were identical. Therefore, we have identified the 35K-TBP as the nucleotide-activated T_3 -binding entity present in the cytosol of cultured astroglial cells.

The molecular mass of the astroglial-soluble T_3 -binding protein, calculated from the sedimentation coefficient and the R_s , is approx. 65 kDa. The 35K-TBP is a subunit of this protein, but

it is not known whether the native protein is an homodimer of 35 kDa subunits, or is formed of one 35 kDa subunit and other subunit(s). Two-dimensional electrophoresis showed that there is only one isoform of the 35K-TBP, of pI approx. 5. However, it is not known to what extent covalent attachment of T_3 to its binding site has altered the pI of labelled 35K-TBP.

In brain [28], kidney [13] and liver [29], the identification of the T_3 -binding proteins was based on purification of the T_3 -binding activity and determination of the molecular mass of the purified proteins. None of them was identified by affinity labelling. These proteins were reported to be a 58 kDa monomer in kidney and brain, and a homodimer of 38 kDa subunits in liver. It is possible that different cytosolic proteins participate in T_3 binding in different tissues or cells. However, it is also possible that the 35K-TBP identified in astroglial cells is present in other cell types, and co-purified with other T_3 -binding polypeptides: our unpublished studies show the photolabelling of a 35 kDa polypeptide in rat brain cytosol in the presence of $NADP^+$ and NADPH.

The molecular mass of the 35K-TBP is in the same range as those of several cytosolic proteins which bind low-molecular-mass ligands. These include a 32 kDa α -tocopherol transfer protein from rat liver [5], a 34 kDa pregnenolone-binding protein from guinea-pig adrenal cortex [1], a 35 kDa phosphatidylinositol transfer protein from human leukaemia cells [6] and a 38 kDa methotrexate-binding protein from murine leukaemia cells [30]. It is not known whether the 35K-TBP is structurally related to some of these cytosolic proteins.

In astroglial cell cytosol, NADPH could act either as an allosteric activator of T_3 binding or as a cofactor involved in an enzymic reduction step resulting in activation of T_3 binding. The second hypothesis is unlikely, since only partial activation was promoted by low levels of NADPH, despite the endogenous $NADP^+$ -reducing activity or the addition of an exogenous $NADP^+$ -reducing system (glucose 6-phosphate + glucose-6-phosphate dehydrogenase), which continuously replenished the NADPH pool. Saturation analysis of T_3 binding revealed the presence of a single class of high-affinity sites, in the presence of NADPH. Their affinity was decreased, without modification of their number, when NADPH concentrations were lowered, indicating a conformational change in the T_3 -binding protein.

The ED_{50} of binding activation by NADH (which, according to the manufacturer, is not contaminated by NADPH, even in trace amounts) is about 3000 times higher than the ED_{50} of binding activation by NADPH. This indicates that the 2'-phosphorylated group is essential for pyridine nucleotide binding to its regulatory site. Since NAD^+ neither activates T_3 binding nor prevents activation by other pyridine nucleotides, it is likely that it does not interact with the activation site.

In the present work we demonstrate that only the reduced forms of the pyridine nucleotides (NADPH and NADH) do activate the T_3 -binding protein in astroglial cell cytosol. The characteristics of T_3 -binding activation by nucleotides in purified preparations from kidney [13], brain [28], and liver [29] differ from those found in the present work. NADPH and $NADP^+$ activated brain binding sites with similar ED_{50} (approx. 1×10^{-9} M), but NADPH was 1000 times less potent (ED_{50} approx. 5×10^{-6} M) than $NADP^+$ in activating kidney binding sites. Finally, liver binding sites were activated by low concentrations of NADPH (ED_{50} approx. 3×10^{-9} M), but not by $NADP^+$, which inhibited the NADPH-induced activation. The differences from the present work may be due to cell-type specificity, or because purified preparations may lack some of the enzymic activities and endogenous redox cofactors present in the crude cytosol.

Two non-receptor cytosolic steroid-binding proteins are also

activated by nucleotides: 3',5'-ADP activated the pregnenolone-binding protein from the adrenal cortex [31], and NADH activated the binding of the synthetic androgen methyltrienolone to a protein from human placenta [2]. Whether some of these cytosolic proteins are functionally related to the 35K-TBP awaits further studies.

The characteristics that distinguish the soluble 35K-TBP from the nuclear T_3 receptors are their intracellular location, their activation by reduced pyridine nucleotides, their subunit molecular mass and their specificity of iodothyronine binding. Indeed, the nuclear receptors bind triac and ip- T_3 as well as, or more strongly than, T_3 [7], whereas these analogues have 15- and 90-fold lower affinity than T_3 for the 35K-TBP. The affinity of the soluble 35K-TBP for T_3 , in the presence of NADPH, is of the same order of magnitude as that of nuclear T_3 receptors isolated from glial cells [32]. However, the number of 35K-TBP binding sites is > 200 times larger than that of nuclear binding sites measured in intact cultured astroglial cells [15]. This suggests that, under normal cellular redox conditions (i.e. an approx. 70-fold excess of free NADPH over free $NADP^+$ [33]), the 35K-TBP was the major high-affinity T_3 (and T_4) binding protein in astroglial cells.

Removing free T_3 from cytosol, by DCC adsorption in the presence of NADPH, resulted in a half-time of T_3 dissociation which was 5 times shorter than that promoted by an exogenous NADPH oxidation system. In the latter case, the rate-limiting step of T_3 dissociation may be NADPH dissociation from its site of activation (or its oxidation at this site), since free NADPH oxidation is almost immediate. We have shown that the NADPH oxidation system not only prevented T_3 binding from occurring, but also promoted the release of bound T_3 in the absence of any change in the total concentration of the hormone. This strongly suggests that an important role of the 35K-TBP is sequestration/release of intracellular thyroid hormones, and that this function is regulated by the redox state of astroglial cell compartment(s).

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