THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 265, No. 9, Issue of March 25, pp. 5296-5302, 1990
Printed in U.S.A.

Thyroxine-dependent Modulation of Actin Polymerization in Cultured Astrocytes

A NOVEL, EXTRANUCLEAR ACTION OF THYROID HORMONE*

(Received for publication, November 1, 1989)

Catherine A. Siegrist-Kaiser‡§, Christiana Juge-Aubry§, Mary P. Tranter¶, Deborah M. Ekenbarger‡, and Jack L. Leonard‡‖

From the ‡Departments of Physiology and Nuclear Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01655, §Thyroid Research Unit, Endocrine Division, Department of Medicine, University of Geneva, Geneva, Switzerland, and the ¶Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, Massachusetts 02115

Actin depolymerization specifically blocks the rapid thyroid hormone-dependent inactivation of type II iodothyronine 5′-deiodinase. Thyroid hormone appears to regulate enzyme inactivation by modulating actin-mediated internalization of this plasma membrane-bound protein. In this study, we examined the interrelationships between thyroxine-dependent enzyme inactivation and the organization of the actin cytoskeleton in cultured astrocytes. Steady-state enzyme levels were inversely related to actin content in dibutyryl cAMP-stimulated astrocytes, and increases in filamentous actin resulted in progressively shorter enzyme half-lives without affecting enzyme synthesis. In the absence of thyroxine, filamentous actin decreased by ~40% and soluble actin correspondingly increased; thyroxine normalized filamentous actin levels without changing total cell actin. Thyroxine treatment for only 10 min resulted in an ~50% loss of enzyme and increased filamentous actin 2-fold. Neither cycloheximide nor actinomycin D affected the thyroxine-induced actin polymerization. Astrocytes grown without thyroxine also showed a disorganized actin cytoskeleton, and 10 nM thyroxine or 10 nM reverse triiodothyronine normalized the actin cytoskeleton appearance within 20 min; 10 nM 3,3′,5-triiodothyronine had no effect. These data show that thyroxine modulates the organization of the actin cytoskeleton in astrocytes and suggest that regulation of actin polymerization may contribute to thyroid hormone's influence on arborization, axonal transport, and cell-cell contact in the developing brain.

Thyroid hormone plays an essential role in the growth and development of the mammalian brain influencing such diverse events as dendritic arborization, myelinization, expression of key enzymes in neurotransmitter synthesis, and the glial/neuronal cell ratio (1, 2). However, specific effects of thyroid hormone excess or deficiency on cell metabolism in the adult brain have been elusive, even though the presence of substantial numbers of nuclear thyroid hormone receptors is well documented in brain (3–5). Since most, if not all, of the effects of thyroid hormone are thought to be initiated by the selective binding of 3,5,3′-triiodothyronine (T_3)[1] to these receptors, the inability of the adult brain to respond to thyroid hormone remains poorly understood.

In most animals the bioactive form of thyroid hormone, T_3, is derived from the enzyme-catalyzed 5′-deiodination of T_4, the major secretory product of the thyroid gland (6). This enzyme-catalyzed bioactivation of thyroid hormone accounts for >75% of the T_3 found in the circulation (7, 8). The brain differs from other hormone-responsive tissues in that it relies almost exclusively on intracellular conversion of T_4 to T_3 rather than the circulation, as its source of bioactive T_3 (6–8). Interestingly, the activity of type II iodothyronine 5′-deiodinase, the brain enzyme catalyzing this reaction, shows rapid 5- to 10-fold increases after thyroidectomy and equally rapid decrements with hormone replacement (9–11). This ability of the brain to modulate type II iodothyronine 5′-deiodinase content and thus intracellular T_3 production appears to be a homeostatic mechanism to preserve intracerebral T_3 levels within narrow limits and is likely to play a role in preventing pathophysiological changes in cerebrocortical metabolism in hyper- or hypothyroidism (10).

The mechanism(s) by which thyroid hormone regulates levels of this plasma membrane-bound enzyme in the brain have been studied both *in vivo* and in cell culture and do not appear to involve the classical nuclear receptor. Comparison of the ability of individual iodothyronines to diminish cerebrocortical type II iodothyronine 5′-deiodinase activity in hypothyroid rats showed $T_4 \approx rT_3 \gg T_3$ (10, 11), in sharp contrast to the rank order of potency for binding to the nuclear T_3 receptor ($T_3 \gg T_4 > rT_3$). Thyroid hormone-induced increases in enzyme inactivation/degradation are unaffected by inhibitors of transcription or translation and can be accounted for by a selective increase in enzyme degradation (12). Comparable results have been obtained in cell culture models using dispersed fetal (13) or neonatal rat brain (14, 15), the neuroblastoma cell line NB41A3 (16), and the Bt_2cAMP-stimulated astroglial cell (17, 18).

The cellular events mediating this thyroid hormone response remain to be established. Ligand-induced enzyme in-

* This work was supported by National Institutes of Health Grant DK38 772 (to J. L. L.) and the Swiss National Research Foundation Grant 3.943.0.84. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom reprint requests should be addressed.

[1] The abbreviations used are: T_3, 3,5,3′-triiodothyronine; bt_2cAMP, dibutyryl cyclic AMP; NBD-phallacidin, 7-nitrobenz-2 oxa-1,3-diazole-phallacidin; DMEM, Dulbecco's modified Eagle's medium; PMSF, phenylmethylsulfonylfluoride; F-actin, filamentous actin; G-actin, globular (soluble) actin; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; T_4, thyroxine; rT_3, 3,3′5′-triiodothyronine; 3,3′T_2, 3,3′-diiodothyronine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

activation has been proposed to account for the ability of substrates and competitive enzyme inhibitors to decrease type II iodothyronine 5′-deiodinase activity in NB41A3 cells (16), although the molecular mechanism(s) for this inactivation remains undefined. We have recently shown that the T_4-dependent increase in enzyme inactivation is energy-dependent and that cytochalasins specifically block enzyme loss (18). In this study, we determined the effects of thyroid hormone on actin content and microfilament organization in astrocytes and examined some of the interrelationship(s) between the T_4-dependent regulation of this short-lived membrane-bound enzyme and the actin cytoskeleton. The results demonstrate that the organization of the actin cytoskeleton in astrocytes is dynamically regulated by thyroid hormone. In addition, T_4's influence on the polymerization state of the actin cytoskeleton is temporally related to the T_4-induced changes in type II 5′-deiodinase.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium, antibiotics, Hank's salt solution, glucose, trypsin, and fetal bovine serum (heat-inactivated) were obtained from GIBCO. Culture flasks and plasticware were purchased from Nunc. L-T_4, L-T_3, bt_2cAMP, and hydrocortisone were from Sigma; L-rT_3 was from Calbiochem; and L-3,3′-T_2 was from Henning GmbH. NBD-phallacidin was obtained from Molecular Probes, Inc. $Na^{125}I$(~17 Ci/mg) was purchased from Du Pont-New England Nuclear. L-[3′- or 5′-^{125}I]rT_3 (~2200 Ci/mmol) was prepared by the method of Weeke and Orskov (19) and purified to >95% as described previously (17, 18) with $^{125}I^-$ as the major contaminant. Pregnant Sprague-Dawley rats were bred in the Animal Facilities at the University of Massachusetts or the Universite de Geneve. All other reagents were of the highest purity commercially available.

Cell Culture Conditions—Astroglial cells were prepared and grown as described previously (17). Cultures were fed every 2–3 days and were subcultured (20–30 × 10^3 cells/cm^2) every 7–10 days; in all experiments, cells were used between the 2nd and 6th passage.

Actin content in astrocyte cultures was altered by varying the initial plating density of the cells as described (29). Primary dispersions of glial cells were plated at increasing density and grown to confluence. Thereafter, all cells were subcultured at 1 × 10^4 cells/cm^2 for two passages.

Thyroid hormone levels in the culture medium were altered by replacing the serum supplement with individual iodothyronines dissolved in Hank's solution containing bovine serum albumin (final concentration 1 mg/ml in the culture medium). Iodothyronines were added in the presence of BSA to permit the free hormone levels to be accurately determined and to prevent nonspecific adsorption of the iodothyronine to the culture flask. "Euthyroid" medium consisted of DMEM containing 10% fetal bovine serum or serum-free DMEM containing 10 nM T_4 and 1 mg/ml BSA (670 pM free T_4 as determined by equilibrium dialysis). "Hypothyroid" medium consisted of serum-free DMEM, lacking thyroid hormones. Cells were grown in hypothyroid medium for 24–48 h prior to enzyme induction. Type II 5′-deiodinating activity was induced by treating cells with 0.5 mM bt_2cAMP and 100 nM hydrocortisone for 16 h as described previously (17, 18).

Kinetics of Enzyme Inactivation—Steady-state levels of type II iodothyronine 5′-deiodinase were induced as described above. At the start of the experiment, protein synthesis was blocked with 0.1 mM cycloheximide, and triplicate flasks (25 cm^2) of confluent cells were harvested at the times indicated. Type II iodothyronine 5′-deiodinase activity was then measured in cell sonicates as described previously (18).

The time course of T_4's effect on type II iodothyronine 5′-deiodinase inactivation was studied by adding 10 nM T_4 plus 1 mg/ml BSA (final concentrations) to bt_2cAMP-stimulated cells in serum-free medium. Triplicate flasks were harvested at timed intervals and enzyme activity measured in cell sonicates.

Determination of Cell Actin Content and Isolation of the Astroglial Cytoskeleton—Total actin and filamentous (F-) actin were determined by Western blot analysis (20). Cell monolayers (25-cm^2 flasks) were washed free of culture medium with ice-cold 20 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl, treated for 5 min with 500 μl of 50 mM Tris buffer (pH 6.8) containing 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100 (21), and scraped from the dish. 50 μl of the cell suspension was then mixed with 10 μl of 5 × PAGE sample buffer (250 mM Tris buffer (pH 6.8), 5% (w/v) SDS, 700 mM β-mercaptoethanol, 50% (v/v) glycerol, 0.005% (w/v) bromophenol blue) and kept for later analysis.

The cell cytoskeleton was collected as the Triton-insoluble fraction from the remaining 450 μl of cell suspension by centrifugation at 12,000 × *g* for 10 min at 4 °C (21). Pellets were resuspended in 450 μl of 50 mM Tris buffer (pH 6.8) containing 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 0.5% Triton X-100. Equal volumes (20-μl aliquots) of the cell suspension, Triton-soluble supernatant (containing G-actin), and resuspended Triton pellet (containing F-actin) were separated on 12.5% SDS-PAGE gels, transferred to nitrocellulose by electroblotting at 60 V for 60 min, and β-actin-identified with anti-actin IgG (Biomedical Tech. Inc.). Immune complexes were identified with protein G-gold conjugate and silver-enhanced according to the manufacturer's instructions (Bio-Rad) and quantitated by scanning densitometry.

Triton-insoluble cytoskeleton proteins were also analyzed by SDS-PAGE. Proteins were dissolved in PAGE sample buffer composed of 50 mM Tris buffer (pH 6.8), 1% (w/v) SDS, 140 mM β-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromphenol blue, denatured by heating in a boiling water bath for 10 min, and separated by electrophoresis on 12.5% SDS-PAGE according to Laemmli (22). Gels were stained with Coomassie Blue (R-250), dried, and protein profiles scanned by laser densitometer.

NBD-phallacidin Staining of the Actin Cytoskeleton—Astrocytes were seeded onto polylysine-coated glass coverslips and grown for 1–3 days in serum-containing medium. Twenty-four hours prior to enzyme induction, the medium was replaced with either serum-free, serum-containing, or iodothyronine-supplemented medium and type II iodothyronine 5′-deiodinase activity induced by treatment for 16–18 h with 0.5 mM bt_2cAMP and 100 nM hydrocortisone (18). Cells were then treated with 10 μM colchicine for 60 min to relax the contracted cell borders typical of bt_2cAMP-stimulated astrocytes. Short-term exposure to colchicine has no effect on either the induction or inactivation of type II 5′-deiodinase (18). Iodothyronines were then added for 20 min as indicated, and the cells were fixed for histochemical identification of F-actin.

Cells were fixed with 3.7% formaldehyde in PBS for 30 min, washed three times with PBS, permeabilized with acetone at −20 °C for 10 min, and air-dried. F-actin was stained with 0.3 μM NBD-phallacidin (Molecular Probes, Inc., Junction City, OR) for 30 min at room temperature. After washing with PBS, coverslips were mounted onto glass slides in 50% glycerol in PBS (pH 7.8) and examined using a Zeiss microscope equipped with an epi-optics UV and standard fluorescein isothiocyanate filter set. Micrographs were taken with high-speed Ektakrome ASA 400 film.

Analytical Procedures—Total RNA was prepared by the method of Chirgwin *et al.* (23), separated on 1.4% agarose/formaldehyde gels, and transferred to nitrocellulose by diffusion blotting. Blots were probed at 42 °C for 16 h with 10 ng/ml ^{32}P-labeled β-actin cDNA prepared by nick translation (24) in a hybridization solution composed of 50% (v/v) formamide, 1 mg/ml Ficoll 400, 1 mg/ml polyvinylpyrrolidone, 1 mg/ml BSA, 750 mM NaCl, 75 mM sodium citrate buffer (pH 7.0), 100 μg/ml sheared salmon sperm DNA, and 1 mg/ml SDS. Nitrocellulose blots were washed at high stringency (30 mM NaCl, 3 mM sodium citrate, 1 mg/ml SDS at 60 °C for 15 min) and exposed to Kodak XAR-5 film at −70 °C. Mouse β-actin cDNA was kindly provided by Dr. Joseph Majzoub, Harvard Medical School.

Type II iodothyronine 5′-deiodinase activity was determined in glial cell sonicates prepared in 10 mM HEPES buffer (pH 7.0), containing 1 mM EDTA and 10 mM dithiothreitol. Enzyme activity was determined by measuring the release of radioiodide from 2 nM L-[3′- or 5′-^{125}I]rT_3, in the presence of 20 mM dithiothreitol and 1 mM propylthiouracil as described previously (12, 25). Under these experimental conditions, equivalent amounts of iodide and 3,3′-T_2 are produced. Enzymatic activity is reported as femtomoles of iodide released per h.

Miscellaneous Methods—Protein was determined by the method of Bradford (26) using human γ-globulin as the protein standard. DNA was measured by the method of Hill and Whatley (27).

RESULTS

Thyroxine has been shown to regulate type II iodothyronine 5′-deiodinase levels by modulating the rate of enzyme degradation without altering enzyme synthesis. Cytochalasins se-

lectively arrest this process (18). Since type II iodothyronine 5′-deiodinase is a short-lived integral membrane protein, associated with neurilemmal membranes (28), then a likely initial step in the degradation pathway is enzyme internalization. To determine whether the steady-state levels and/or the $t_{½}$ of type II iodothyronine 5′-deiodinase are influenced by the cell actin content, we varied the actin content of astrocytes by altering the plating density of the primary dispersions as described by Goldman and Chiu (29). Depicted in Fig. 1*A* is the effect of initial plating density on type II iodothyronine 5′-deiodinase inactivation. The rate of enzyme disappearance in cycloheximide-blocked cultures was inversely related to the initial plating density, being fastest in cells derived from the lowest initial density and slowest in cells grown from dispersions plated at the highest density.

Shown in Fig. 1*B* are the effects of increasing the initial plating density on composition of the Triton-insoluble cytoskeleton in astrocytes. As expected, F-actin was most abundant in cells derived from primary dispersions seeded at low density and decreased proportionally as the initial plating density increased. These changes in F-actin content were maintained through three passages indicating the density of the primary dispersion was a prime factor in determining actin levels in astrocytes. In contrast, the intermediate filament proteins, GFAP and vimentin, were much less dependent upon initial plating density with the apparent changes in quantity shown in *lanes a–c* (Fig. 1*B*) due to loading of equivalent amounts of protein on the SDS-PAGE gel.

A comparison of the effects of altered actin concentration on type II iodothyronine 5′-deiodinase activity and $t_{½}$ is summarized in Table I. Steady-state enzyme levels increased proportionately from 43 to 123 units/mg protein and the $t_{½}$ increased >3-fold in cells grown from dispersions in which the plating density was increased 20-fold. These increases in type II iodothyronine 5′-deiodinase were due primarily to changes in the inactivation rate since enzyme production rates, as calculated from the steady-state enzyme levels and the disappearance rate constants (k), were relatively unchanged at 349 ± 34 (S.E., n = 4) units/mg protein/h in the cells obtained from the four different initial plating densities.

F-actin levels were also inversely related to the initial plating density and remained so for at least three passages, decreasing ~4-fold as the plating density increased 20-fold (Table I). These changes in F-actin paralleled the increase in total cell actin, thus the ratio of F- to G-actin remained constant (data not shown).

Effects of T_4 on Astrocyte Actin Content, mRNA, and Composition—T_4 had a marked effect on the actin content of the Triton-insoluble cytoskeleton of astrocytes. Astrocytes grown in the thyroid hormone-deficient medium (serum-free medium) had only 40% of the F-actin of cells grown in serum-supplemented medium (8.1 *versus* 12.8% of the total Triton-insoluble protein), and addition of 10 nM T_4 restored the F-

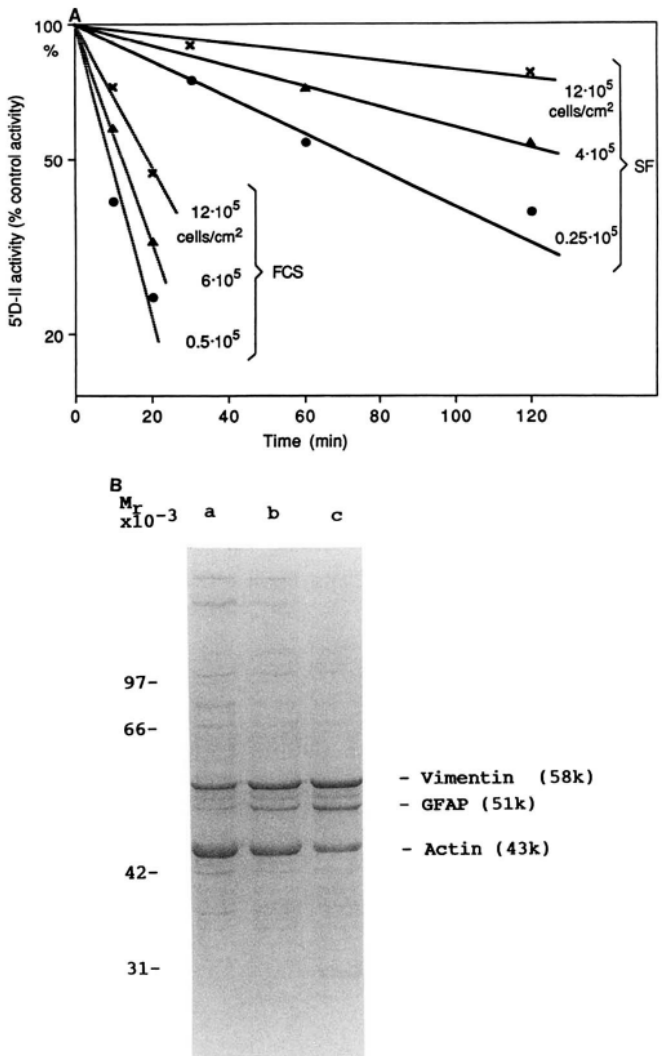

FIG. 1. **Effects of initial plating density on the rate of type II iodothyronine 5′-deiodinase inactivation and the composition of the Triton-insoluble cytoskeleton in bt_2cAMP-stimulated astrocytes.** *A*, the rate of type II iodothyronine 5′-deiodinase (*5′D-II*) inactivation was determined in triplicate (25-cm² flasks) in cells at passage three as described under "Materials and Methods." Data are presented as the percent of the starting enzyme activity of closely agreeing (±10%) triplicate cultures. *SF*, serum-free culture conditions; *FCS*, serum containing culture conditions. *B*, SDS-PAGE of the Triton-insoluble cytoskeleton in astrocytes. Aliquots (15 μg of protein) of the Triton-insoluble pellets obtained from cells at passage three were separated on 12.5% acrylamide slab gels as described under "Materials and Methods." *Lane a*, cells derived from primary cultures plated initially at 50,000 cells/cm²; *lane b*, cells plated at 400,000 cells/cm²; *lane c*, cells plated at 1,200,000 cells/cm².

TABLE I

Steady-state type II iodothyronine 5′-deiodinase levels, $t_{½}$, and F-actin content in bt_2cAMP-treated astrocytes derived from primary dispersions plated at increasing density

Quadruplicate flasks (25 cm²) of confluent cells were grown from cultures seeded at increasing initial plating density and subcultured three times. Type II iodothyronine 5′-deiodinase was induced by treating the cells for 16 h with 0.5 mM bt_2cAMP, 100 nM hydrocortisone. Enzyme half-times were determined from linear semilogarithmic plots of the disappearance of type II catalytic activity in cycloheximide-blocked cells *versus* time as described under "Materials and Methods." F-actin content was determined as described under "Materials and Methods" and is expressed as AU/mg cell protein (AU = arbitrary absorbance units). Data are from two representative experiments, and results are presented as means of closely agreeing (± 10%) quadruplicate flasks.

Plating density	Type II iodothyronine 5′-deiodinase activity		F-actin
	Steady state	$t_{½}$	
(cells/cm²) × 10⁻⁵	*units/mg protein*	*min*	*AU/mg cell protein*
10	123	17	11 ± 5
5	99	11	22 ± 7
1	64	9	37 ± 13
0.5	43	4.5	49 ± 7
12		17.6	
6		12.5	
0.5		8.36	

actin content to control levels (11.4 *versus* 12.8% of total Triton-insoluble protein). Identical reductions in F-actin content were observed when 5% (v/v) hypothyroid rat serum was substituted for the calf serum in the culture medium (data not shown), indicating that other serum factors had little, if any, influence on F-actin content under these culture conditions. Total cellular actin, GFAP, and vimentin were unaffected by the presence or absence of serum (data not shown).

To examine whether T_4 and/or bt_2cAMP treatment contributed to the changes in F-actin content by stimulating actin transcription, we determined the effects of T_4 and bt_2cAMP on β-actin mRNA levels. As shown in Fig. 2, neither thyroid hormone nor 16 h of bt_2cAMP treatment altered β-actin mRNA levels in astrocytes. Similarly, total actin content in the astrocyte was unaffected by 16 h of T_4 treatment of bt_2cAMP-stimulated cells as judged by Western blot analysis with anti-actin antibodies (230 absorbance units/mg protein *versus* 280 absorbance units/mg protein). These data show that thyroid hormone had little or no transcriptional control of β-actin gene expression.

Rapid Effects of Thyroid Hormone on Inactivation/Degradation of Type II 5′-Deiodinase and Actin Polymerization—Shown in Fig. 3 is the time course for the T_4-dependent decrease of type II 5′-deiodinase in astrocytes. Addition of T_4 (~100 pM "free" hormone) to bt_2cAMP-treated cells grown in serum-free medium resulted in a rapid 50% loss of activity during the first 8 min followed by a progressively slower loss of catalytic activity that plateaued at 35–40% of the serum-free control. The rapid effects of T_4 on the F-actin content of astrocytes are shown in Table II. F-actin levels followed a similar time course to that of type II iodothyronine 5′-deiodinase, with a rapid ~2-fold increase during the first 5–10 min and a plateau after ~10 min of treatment. Proportional decreases in G-actin were observed with no change in total actin content during this short period of T_4 treatment (data not shown). Neither cycloheximide nor actinomycin D pretreatment blocked the T_4-induced increases in F-actin in astrocytes indicating that neither translation nor transcription were required for this thyroid hormone effect.

Effects of Thyroid Hormone on Microfilament Organization in Bt_2cAMP-stimulated Astroglial Cells—Because both cyclic

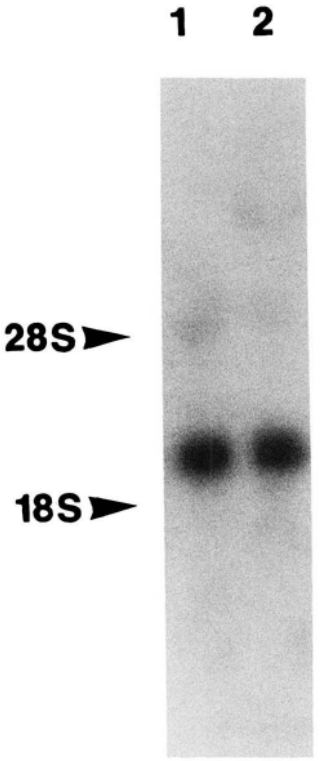

FIG. 2. **β-Actin mRNA levels in cultured glial cells.** Confluent primary cultures (100,000 cells/cm^2 initial plating density) were subcultured at 1×10^4 cell/cm^2 and grown to confluence. Cells were then treated with 0.5 mM bt_2cAMP, 100 nM hydrocortisone for 16 h to induce type II iodothyronine 5′-deiodinase activity as indicated. 10-μg aliquots of total RNA were separated on a 1.2% agarose/formaldehyde gel, transferred to Duralose, and probed with β-actin ^{32}P-cDNA as described under "Materials and Methods." *Lane 1*, 10 μg of RNA from untreated cells grown in 10% serum-containing medium; *lane 2*, 10 μg of RNA from cells grown in serum-free medium and treated with 1 mM bt_2cAMP and 100 nM hydrocortisone for 16 h.

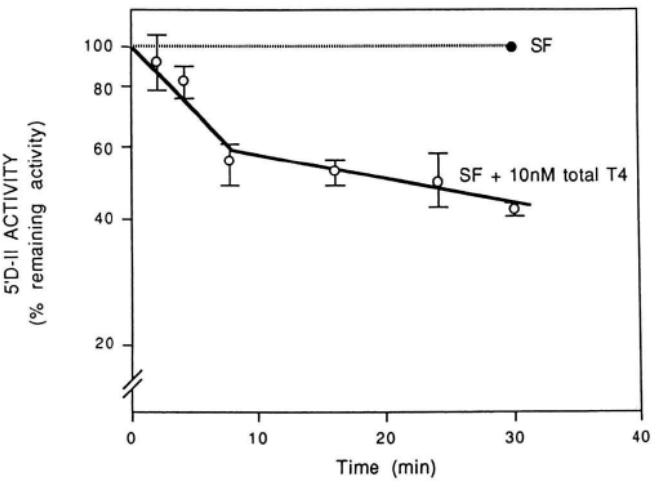

FIG. 3. **Rapid effects of T_4 on inactivation of type II 5′-deiodinase (*5′D-II*).** Astrocytes were grown in serum-free medium and stimulated as described under "Materials and Methods." T_4 (final concentration 10 nM in Hank's solution containing 1 mg/ml BSA) was added at the start of the experiment and triplicate flasks of cells harvested at the times indicated. Type II 5′-deiodinating activity was determined in an aliquot of the cell sonicates as described under "Materials and Methods" and the results reported as the percent of starting enzyme activity. Data are reported as the means of closely agreeing (±10%) triplicate determinations.

TABLE II

Effects of T on F-actin content of bt_2cAMP-stimulated astrocytes in absence or presence of cycloheximide or actinomycin D

Confluent cultures (25 cm^2; seeded at 4×10^5 cells/cm^2) were treated for 16 h with 0.5 mM bt_2cAMP and 100 nM hydrocortisone to induce type II iodothyronine 5′-deiodinase. Flasks were then pretreated for 15 min with PBS, 0.1 mM cycloheximide, or 10 μM actinomycin D. 10 nM T_4 was then added as indicated and triplicate flasks harvested at 0, 5, 10, and 20 min. F-actin was determined on the cells from individual flasks as described under "Materials and Methods." Data are expressed as arbitrary absorbance units (AU) per mg of total cell protein and are reported as the means of closely agreeing (±10%) triplicates.

Pretreatment	Time	T_4	F-actin
	min		*AU/mg total cell protein*
None	0		6.6
	20		7.6
	5	+	10.4
	10	+	16.4
	20	+	14.3
Cycloheximide	5	+	14
	10	+	14.6
	20	+	18.3
Actinomycin D	5	+	12.1
	10	+	19.4
	20	+	15.5

nucleotides and cytochalasins promote structural reorganization of actin (30), and cytochalasins antagonize the T_4-dependent regulation of type II iodothyronine 5′-deiodinase (18), we examined the effects of thyroid hormone on microfilament organization in astrocytes (Fig. 4). A complex network of actin bundles was present in astrocytes grown in serum-containing medium (Fig. 4*a*), and this network was markedly reduced in both amount and fiber length in the absence of thyroid hormone (Fig. 4*b*). T_4 replacement alone was sufficient to restore the organization of the actin cytoskeleton (Fig. 4*c*) to that observed in control cultures.

The rapid effects of iodothyronine replacement on the appearance of the astrocyte actin cytoskeleton are illustrated in *panels d–f* of Fig. 4. Cells exposed to 10 nM T_3 for 20 min showed little or no reorganization of their actin cytoskeleton (Fig. 4*d*), similar to T_3's lack of effect on enzyme inactivation at these concentrations (10, 18). In contrast, cells treated for only 20 min with either 10 nM T_4 (Fig. 4*e*) or 10 nM rT_3 (Fig. 4*f*) showed nearly complete re-polymerization of the actin cytoskeleton, a time course comparable with the hormone-induced changes in the type II iodothyronine 5′-deiodinase inactivation shown in Fig. 3.

DISCUSSION

The ability of cytochalasins to block the T_4-dependent inactivation of type II iodothyronine 5′-deiodinase (18) raised the possibility that there was a functional relationship between the organization of the actin cytoskeleton and the inactivation of this membrane-bound enzyme. In this study, we have shown that (i) increases in the cell actin content were accompanied by decreases in the $t_{½}$ of type II iodothyronine 5′-deiodinase with no change in the enzyme production rate; (ii) T_4 and rT_3 promoted F-actin formation and increased the rate of enzyme inactivation, whereas T_3 was ineffective; (iii) the time courses for T_4-dependent formation of F-actin and the increase in type II iodothyronine 5′-deiodinase turnover were the same; and (iv) both the T_4-dependent increase in type II iodothyronine 5′-deiodinase inactivation and actin

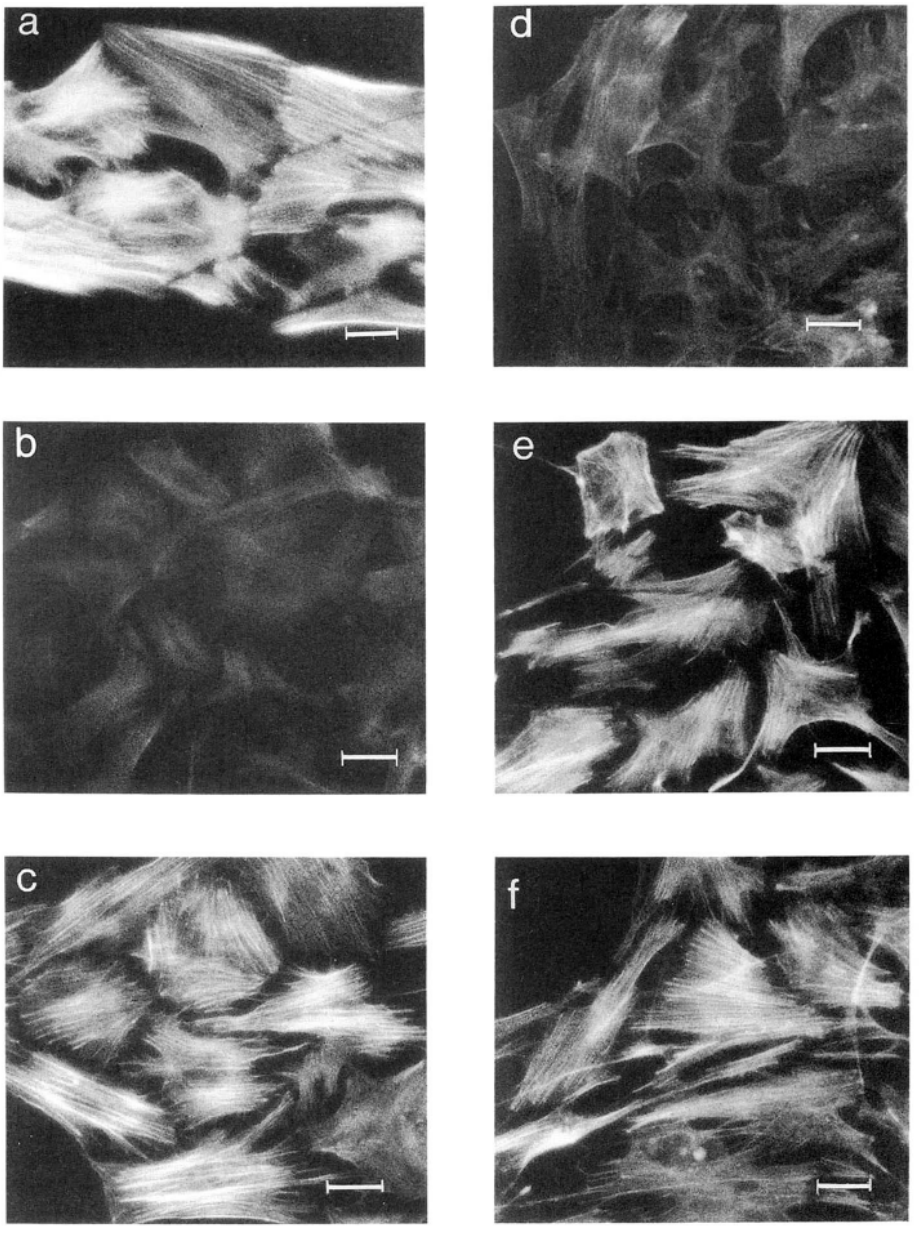

FIG. 4. **NBD-phallacidin-stained F-actin in astrocyte cultures.** Astrocytes were grown on coverslips in serum-supplemented medium. Forty-eight hours prior to the staining, the culture medium was changed to serum-supplemented (*a*), serum-free medium (*b*), and serum-free medium supplemented with 10 nM T_4 plus 1 mg/ml BSA (*c*). All cultures were stimulated for 16 h with 1 mM bt_2cAMP and 100 nM hydrocortisone, followed by a 1-h incubation with 10 μM colchicine to relax the contracted cell borders. The actin cytoskeleton was stained with NBD-phallacidin as described under "Materials and Methods." Scale bar = 10 μm. *Panels d–f* show serum-free cultures treated for 20 min prior to fixation with 10 nM T_3, 10 nM T_4 or 10 nM rT_3, respectively.

polymerization did not require continued protein synthesis or transcription. These data suggest that the extranuclear T_4-dependent modulation of type II 5′-deiodinase depends, in part, upon alterations in the polymerization state of the actin cytoskeleton.

Actin comprises a major fraction of the astrocyte cytoskeleton *in vivo* (31) and in cultures of immature and mature astrocytes (32). The ability to alter the actin content of cultured astrocytes by changing the initial plating density (29), and the observation of Ciesielski-Treska *et al.* (32) that a 24-h exposure to bt_2cAMP had no effect on total actin content in these cells, allowed us to manipulate actin levels in cells expressing type II iodothyronine 5′-deiodinase. Steady-state enzyme levels decreased and the $t_{½}$ of the enzyme grew progressively shorter as the actin content increased in the cultured cells. Interestingly, changes in the total actin content of glial cells had no effect on the rate of type II iodothyronine 5′-deiodinase synthesis suggesting that alterations in the composition of the cytoskeleton were responsible for the changes in the inactivation/degradation of this enzyme.

A relationship between the T_4-dependent modulation of the biological half-life of type II iodothyronine 5′-deiodinase and cell actin levels was further strengthened by examination of thyroid hormone's influence on F-actin content. Cells grown in the absence of thyroid hormone contained ~40% less F-actin than cells grown in presence of T_4. Addition of T_4 alone normalized F-actin in cells grown in serum-free medium suggesting that thyroid hormone was essential for normal actin polymerization. Importantly, the T_4-dependent changes in F-actin content were not mediated by hormone-induced changes in transcription or translation, since neither cycloheximide nor actinomycin D affected this cell response. In addition, β-actin mRNA levels were unaffected by T_4. These data are consistent with the proposed extranuclear site of action for thyroid hormone (12, 18).

Examination of the actin cytoskeleton in cultured astrocytes confirmed thyroid hormone's influence on the microfilament network. Astrocytes grown in the absence of thyroid hormone had a poorly developed actin cytoskeleton compared with cultures grown in medium supplemented with serum or with T_4 alone. Iodothyronines induced a rapid reorganization of the poorly developed actin cytoskeleton in cells grown in serum-free medium, requiring as little as 20 min of exposure to thyroid hormone. Both T_4 and the metabolically inactive metabolite, rT_3, promoted repolymerization of the actin cytoskeleton, while an equimolar concentration of T_3 was ineffective. These results are identical to earlier reports of the effects of these iodothyronines on type II 5′-deiodinase turnover *in vivo* (10, 11) and in the cultured astrocyte (18).

The specific events that mediate thyroid hormone's regulation of actin polymerization remain to be established. The finding that this hormone action is not transcriptionally mediated (18, 33) suggests that T_4 may bind to and modulate the activity of one or more actin-binding proteins. Several actin-binding proteins capable of depolymerizing actin filaments or anchoring the actin filament to the membrane have been identified in the brain (34–36) and these may be targets for T_4 binding and subsequent dissociation from the arrested actin fibril.

T_4-dependent changes in actin polymerization may be particularly important during brain development. Both neurite outgrowth and dendritic spine formation are developmentally programmed events that are highly correlated with an increase in actin filaments (37–39). These processing events depend, in part, on specific interactions between the actin cytoskeleton, integrins in the neuronal cell membrane (40–43), and the neurite-promoting domain of the laminin (44). Thus, the ability of thyroid hormone to modulate actin polymerization provides an attractive model by which this hormone could regulate neuronal process formation and ultimately cell-cell interactions.

The recent work of Faivre-Sarrailh and Rabie (33) is in agreement with this idea. They reported that the cerebellum of congenitally hypothyroid rats showed 60–70% decrements in F-actin content with no change in total actin pool and that T_4 administration normalized the F-actin content of the cerebellum. These findings obtained in intact rats parallel our observations on the T_4-dependent changes in actin polymerization in the cultured astrocyte and demonstrate that this culture model will be useful in unraveling the molecular events that mediate the T_4-dependent regulation of actin polymerization in the central nervous system.

Acknowledgments—We would like to thank Professor J. H. Saurat, Dermatology Department, University of Geneva, for providing the fluorescence microscope and Professor Gabbiani, Department of Pathology, University of Geneva, for the use of the laser densitometer. Debra Mullikin-Kilpatrick is thanked for her many helpful discussions.

REFERENCES

1. Ford, D. H., and Cramer, E. B. (1977) in *Thyroid Hormones and Brain Development* (Grave, G. D., ed) pp. 1–18, Raven Press, New York
2. Dussault, J. H., and Ruel, J. (1987) *Annu. Rev. Physiol.* **49,** 321–334
3. Oppenheimer, J. H., Schwartz, H. L., and Surks, M. I. (1974) *Endocrinology* **95,** 897–903
4. Valcana, T., and Timiras, P. S. (1978) *Mol. Cell. Endocrinol.* **11,** 31–41
5. Kolodny, J. M., Larsen, P. R., and Silva, J. E. (1985) *Endocrinology* **116,** 2019–2028
6. Leonard, J. L., and Visser, T. J. (1986) in *Thyroid Hormone Metabolism* (Hennemann, G., ed) pp. 189–230, Marcel Dekker, New York
7. Silva, J. E., Leonard, J. L., Crantz, F. R., and Larsen, P. R. (1982) *J. Clin. Invest.* **69,** 1176–1184
8. van Doorn, J., van der Heide, D., and Roelfsema, F. (1983) *J. Clin. Invest.* **72,** 1778–1792
9. Leonard, J. L., Kaplan, M. M., Visser, T. J., Silva, J. E., and Larsen, P. R. (1981) *Science* **214,** 571–573
10. Silva, J. E., and Leonard, J. L. (1985) *Endocrinology* **116,** 1627–1635
11. Kaiser, C. A., Goumaz, M. O., and Burger, A. G. (1986) *Endocrinology* **119,** 762–770
12. Leonard, J. L., Silva, J. E., Kaplan, M. M., Mellen, S. A., Visser, T. J., and Larsen, P. R. (1984) *Endocrinology* **114,** 998–1004
13. Leonard, J. L., and Larsen, P. R. (1985) *Brain Res.* **327,** 1–13
14. Cavalieri, R. R., Gavin, L. A., Cole, R., and De Vellis, J. (1986) *Brain Res.* **364,** 382–385
15. Courtin, F., Chantoux, F., and Francon, J. (1986) *Mol. Cell. Endocrinol.* **48,** 167–178
16. St. Germain, D. L. (1986) *Endocrinology* **119,** 840–846
17. Leonard, J. L. (1988) *Biochem. Biophys. Res. Commun.* **151,** 1164–1172
18. Leonard, J. L., Siegrist-Kaiser, C. A., and Zuckerman, C. J. (1989) *J. Biol. Chem.* **265,** 940–946
19. Weeke, J., and Orskov, H. (1973) *Scand. J. Clin. Lab. Invest.* **32,** 357–360
20. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4355
21. Chiu, F.-C., Norton, W. T., and Fields, K. L. (1981) *J. Neurochem.* **37,** 147–155
22. Laemmli, U. K. (1970) *Nature* **227,** 680–685
23. Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
24. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., and Struhl, K. (1987) *Current Protocols in Molecular Biology,* John Wiley & Sons, New York

25. Visser, T. J., Leonard, J. L., Kaplan, M. M., Silva, J. E., and Larsen, P. R. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 5080–5084
26. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
27. Hill, B. T., and Whatley, S. (1975) *FEBS Lett.* **56,** 20–23
28. Leonard, J. L., Rennke, H. R., Kaplan, M. M., and Larsen, P. R. (1982) *Biochem. Biophys. Acta* **718,** 109–119
29. Goldman, J. E., and Chiu, F.-C. (1984) *J. Neurochem.* **42,** 175–184
30. Ciesielski-Treska, J., Ulrich, G., Nensch, C., and Aunis, D. (1984) *Neurochem. Int.* **6,** 533–543
31. Goldman, J. E. (1983) *J. Neurosci.* **3,** 1952–1962
32. Ciesielski-Treska, J., Guerold, B., and Aunis, D. (1982) *Neuroscience* **7,** 509–522
33. Faivre-Sarrailh, C., and Rabie, A. (1988) *Brain Res.* **469,** 293–297
34. Pollard, T. D., and Cooper, J. A. (1986) *Annu. Rev. Biochem.* **55,** 987–1035
35. Bamburg, J. R., Harris, H. E., and Weeds, A. G. (1980) *FEBS Lett.* **121,** 178–182
36. Legrand, Ch., Feraz, C., Clavel, C. A., and Rabie, A. (1986) *Brain Res.* **395,** 231–235
37. Markham, J. A., and Fifkova, E. (1986) *Brain Res.* **392,** 263–269
38. Matus, A., Ackermann, M., Pehling, G., Byers, H. R., and Fugiwara, K. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 7590–7594
39. Nona, S. N., Trowell, S. C., and Cronly-Dillon, J. R. (1985) *FEBS Lett.* **186,** 111–115
40. Cohen, J., Burne, J. F., McKinlay, C., and Winter, J. (1987) *Dev. Biol.* **122,** 407–418
41. Bronner-Fraser, M. (1986) *Dev. Biol.* **117,** 528–536
42. Horwitz, A., Duggan, K., Greggs, R., Decker, C., and Buck, C. A. (1985) *J. Cell. Biol.* **101,** 2134–2144
43. Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F., and Hynes, R. O. (1986) *Cell* **46,** 217–282
44. Engvall, E., Davis, G. E., Dickerson, K., Ruoslahti, E., Varon, S., and Manthorpe, M. (1986) *J. Cell Biol.* **103,** 2457–2465

Thyroxine-dependent modulation of actin polymerization in cultured astrocytes. A novel, extranuclear action of thyroid hormone.

C A Siegrist-Kaiser, C Juge-Aubry, M P Tranter, D M Ekenbarger and J L Leonard

J. Biol. Chem. 1990, 265:5296-5302.