Thyroxine Targets Different Pathways of Internalization of Type II Iodothyronine 5'-Deiodinase in Astrocytes

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In the brain, thyroid hormone dynamically regulates levels of the short-lived plasma membrane protein, type II iodothyronine 5'-deiodinase. In cultured astrocytes, thyroxine modulates deiodinase levels by activating cytoskeletal-plasma membrane interactions that increase the rate of inactivation of the enzyme. Here we characterized the effects of these thyroxine-dependent cytoskeletal interactions upon the route of internalization of the deiodinase by following the intracellular transit of the affinity-labeled substrate-binding subunit of the deiodinase (p29). Thyroxine rapidly induced the inactivation of the deiodinase and initiated the binding of p29 to F-actin. By 40 min, >75% of the p29 had been transported to an endosomal pool, which was followed by dissociation of the F-actin-p29 complex. There was no significant accumulation of p29 in the dense lysosomes seen in the presence of thyroxine. In the absence of thyroxine, p29 was internalized and transported to the dense lysosomes at a rate parallel to the inactivation rate of the deiodinase (t1/2 0.75 and 0.64 h, respectively) without involvement with the microfilaments. These data demonstrate that thyroxine targets type II iodothyronine 5'-deiodinase to an endosomal pool by activating specific protein-F-actin interactions involved in microfilament-mediated intracellular protein trafficking.

Type II iodothyronine 5'-deiodinase (5'D-II) is a short-lived, integral plasma membrane protein that generates >80% of the bioactive T3 present in the cerebral cortex by the phenolic ring deiodination of thyroxine (1). Thyroid hormone, specifically thyroxine, dynamically regulates enzyme levels by altering the enzyme’s biological t1/2 by selectively increasing the inactivation of the enzyme without altering enzyme synthesis (2, 3). These actions of thyroxine occur independently of transcription and/or translation, and thyroxine is >100-fold more potent than the more traditionally bioactive T3 in eliciting these actions (3-9). Using cyclic nucleotide-stimulated cultured astrocytes that express high levels of 5'D-II and exhibit all the regulatory aspects seen in the brain in vivo (4-6), we have shown that thyroxine dynamically regulates 5'D-II activity by promoting interactions between the enzyme and the F-actin stress fibers that lead to inactivation and internalization of the enzyme (5).

Eukaryotic cells selectively transport membrane proteins through various distinct membrane pools by mechanism(s) that are poorly understood. One example of membrane protein internalization is the endocytotic pathway (10-12). While the distinct membrane compartments involved in this pathway have been extensively characterized, the mechanism behind the movement and targeting of internalized vesicles to their respective subcompartments remains obscure. Increasing evidence points toward a major role for the cytoskeleton in the regulation of such intracellular protein trafficking (13-18). While the microtubules are most often associated with the intracellular movement of organelles, microfilaments have recently been shown to have an important role in these processes (17-26).

Thyroxine-mediated turnover of 5'D-II provides an excellent model for the study of the role of the microfilaments in intracellular protein trafficking. For example, cyclic nucleotide-stimulated, thyroxine-deficient astrocytes have elevated 5'D-II levels, lack the radial F-actin stress fibers, and the turnover of 5'D-II is relatively slow (5-7). Addition of thyroxine rapidly restores F-actin stress fibers (<5 min) (7), and 5'D-II levels rapidly fall due to thyroxine-initiated binding of the enzyme to the F-actin stress fibers (5). Using immunocytochemistry and confocal microscopy, we have shown that the plasma membrane-bound 5'D-II is quickly translocated into an intracellular pool and that microtubules do not play a role in this cytoskeletal-mediated 5'D-II translocation (5). However, the destination of the internalized 5'D-II, the identity of the intracellular membrane compartment(s) used during its transit, and the molecular events that mediate the exchange of this membrane protein between the different membrane compartments are unknown.

In this study, we have utilized the thyroxine-stimulated inactivation of 5'D-II in astrocytes to examine the role of the F-actin stress fibers in the internalization pathway(s) of short-lived membrane proteins. Using density gradient centrifugation to follow the intracellular transit of the affinity-labeled substrate-binding subunit of 5'D-II, we show that, after thyroxine stimulates the binding of 5'D-II to F-actin, the microfilaments transport this plasma membrane-bound enzyme to the endosomes. In the absence of thyroxine, 5'D-II is transported to the dense lysosomes without the use of the F-actin cytoskeleton. These data demonstrate that thy...
roxine targets the internalization of type II 5'-deiodinase to an endosomal pool by activating specific protein-F-actin interactions involved in microfilament-mediated intracellular protein trafficking.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pregnant (16-17-day gestation) rats were obtained from Charles-
River Laboratories (Kingston, NY). Thyroxine was purchased from Sigma; 3,5,3'-T2 and T3 were obtained from Henning GmbH. Na[131I] (~17 Ci/mg, carrier-free), [3]I[idifluc human transferrin (0.67 mg/ 
mg) and [3]Iprotei G (15-50 mCi/mg) were purchased from Du Pont-
New England Nuclear. Anti-actin antisera was obtained from 
Biomedical Technologies, Inc., and anti-Na*/K* ATPase subunit 
terisa was a gift from Dr. Thomas Smith (Brigham and Women's 
Hospital, Boston, MA). Nitrocellulose (0.2-μm pore size) was obtained 
from Schleicher and Schuell. SDS was obtained from Gallard-Schles-
inger Industries, Inc. Percoll was obtained from Pharmacia, and 
dithiothreitol and taurodeoxycholate was obtained from Calbiochem. 
Dulbecco's modified Eagle's medium, antibiotics, Hank's solution, 
and 0.25% trypsin were obtained from Gibco, and defined bovine 
calf serum (heat-inactivated) was from HyClone, Inc. Culture flasks 
were obtained from Nunc. All other reagents used were of the highest 
purity commercially available. 

**BrAcT3** was synthesized according to published methods (9). 
BrAc[131I]T3 and [3]I[T]T3 were prepared by ionization of BrAC*T3 or 
3,3'-T2, respectively, using the method of Weeke and Orskov (21). 
Radiolabeled iodothyronines were purified by chromatography on 
Sepharose LH-20 columns and their identities and purities were 
determined by thin-layer chromatography (TLC) in the presence and 
absence of 0.5% taurodeoxycholate. Aliquots were then 
diluted to a total volume of 375 μl in sucrose buffer in the 
presence of 1 mg/ml bovine serum albumin. Cells were harvested by scraping, 
suspended in ice-cold 150 mM sodium chloride, 20 mM sodium phos-
phate buffer, pH 7.4 (Triton buffer), triturated and incubated for 10-15 
min at 37 °C followed by labeling with 5 μCi of [131I]-labeled transferrin 
for 2 h at 4 °C. The transferrin-labeled cells were then washed free of 
unbound radiolabel, and the medium was replaced with warmed 
(37 °C) supplemented DMEM without serum. At the times indicated, 
cells were harvested by scraping in phosphate buffer and suspended 
in 500 μl of sucrose buffer, homogenized with one freeze-thaw 
and, fractionated through 16% and then 8% Percoll gradients 
when they reached confluence (7-10 days). 
Confluent cultures from passages 2-4, containing >95% astrocytes 
(22), were utilized for experiments. Cultures were grown in supple-
mented DMEM without serum for at least 8 h to remove serum 
iodothyronines, followed by a 16-h stimulation period with 1 mM 
BrAcAMP and 100 mM hydrocortisone to increase steady-state levels 
of 5'D-II (14) prior to initiation of experimental procedures.

**Culture Conditions**

Dissociated cerebrocortical cells were prepared from 1-day-old 
natal neurons as described previously (22) and grown in a humidified 
atmosphere of 5% CO2 and 95% air at 37 °C in Dulbecco's modified 
Eagle's medium, 15 mM sodium bicarbonate, 33 mM glucose, 1 mM 
sodium pyruvate, 15 mM HEPES, pH 7.4 (supplemented DMEM) 
with 10% (v/v) defined bovine calf serum, 50 units/ml penicillin 
and 90 μg/ml streptomycin. Culture medium was changed three times 
weekly, and cells were subcultured (2-3 x 10^6 cells/cm²) when they 
reached confluence (7-10 days). 

**Affinity Labeling**

Cells were affinity-labeled with 10 nM BrAc[131I]T3 for 20 min as 
described previously (5). Labeling media was then removed, and cells 
were washed once in Hanks' solution. Affinity-labeled cells were then 
were in supernatants were assayed for subcellular fractionation. 
Cells were resuspended in 250 mM sucrose, 10 mM dithiothreitol, 1 mM 
EDTA, 20 mM HEPES, pH 7.0 (sucrose buffer), and lysed by one freeze-thaw cycle. Homogenates (0.5-1.0 mg of 
protein) were centrifuged (805,000 g-min) through self-forming 16% 
Percoll gradients in 0.25 M sucrose (23) and 0.5-ml fractions collected. 
Fractions of buoyant density 1.034-1.050 g/dl were pooled and re-
fractionated through 8% Percoll gradients. Gradient fractions (0.5 
ml) were collected and sonicated, and aliquots were suspended in 10 
mg/ml SDS, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 10 μg/ 
ml camphor blue, 50 mM Tris-HCl, pH 6.8 (PAGE sample buffer). 
Proteins were denatured in a boiling water bath for 5 min and resolved on 
12.5% SDS-PAGE slab gels according to the method of Laemmli 
(24). Radiolabeled proteins were visualized by radioautography, and 
bands of interest were analyzed by scanning densitometry.

**Enzyme Assays**

Aliquots from fractions obtained above were assayed for subcellular 
marker enzymes. Glucose-6-phosphatase (EC 3.1.3.9, endoplasmic 
reticulum) was assayed by the liberation of inorganic phosphate from 
glucose-6-phosphatase (29). Na*/K* ATPase (EC 3.6.1.3, plasma 
membrane) activity was determined by the ouabain-sensitive hydrolysis 
of ATP (26). Release of inorganic phosphate by glucose-6-phospho-
tase and Na*/K* ATPase was measured at 37000 after incubation with 
Fiske-Subbarow reducer solution (25). β-Glucuronidase (EC 3.2.1.24, 
lysosomes) was assayed colorimetrically by the hydrolysis of phenol-
phosphate by glucuronidase at A595. Antinannan specific activity 
determined in cell sonicates as described previously at 2 mM T3 and 
20 mM dithiothreitol in the presence of 1 mM 6-n-propylthiouracil 
(27). Results are presented as the percent of the total enzymatic 
activity fractionated through the gradient, except where noted.

**Transferrin Receptor Labeling (28)**

Confluent astrocytes grown in medium containing 10% serum were washed free of growth medium with excess Hank's solution. Cells 
were then incubated in supplemented DMEM without serum for 30 
min at 4 °C followed by labeling with 5 μCi of [131I]-labeled transferrin 
for 2 h at 4 °C. The transferrin-labeled cells were then washed free of 
unbound radiolabel, and the medium was replaced with warmed 
(37 °C) supplemented DMEM without serum. At the times indicated, 
cells were harvested by scraping in phosphate buffer and suspended 
in 500 μl of sucrose buffer, homogenized with one freeze-thaw 
and, fractionated through 16% and then 8% Percoll gradients 
as described above. Fractions (0.5 ml) were collected and counted.

**Western Analysis**

Actin and the β-subunit of the Na*/K* ATPase were detected by 
Western analysis (29). Actin polymers were depolymerized at low 
ionic strength in 0.05 mM calcium chloride, 1 mM dithiothreitol, 3 
mM sodium azide, 2 mM Tris-HCl, pH 8.0 (depolymerizing buffer), 
denatured in a boiling water bath for 5 min, and applied directly to 
nitrocellulose using the Minifold II slot blot apparatus (Schleicher 
and Schuell) (30). Blots were blocked for 1 h with 50 ml Tris-HCl, 
0.05% (v/v) Tween-20, 50 mg/ml powdered milk, 2 mM calcium 
chloride, pH 8.0, then probed for 16 h at room temperature with an 
affinity-purified polycyal rabbit antisera for either actin (1:5000 
dilution) or the β-subunit of the Na*/K* ATPase (1:1000 dilution). 
Immune complexes were visualized by radioautography following 
incubation of the blots with [125I]labeled protein G (~200,000 cpm/ 
ml). Quantification of actin and the β-subunit of the Na*/K* ATPase 
was determined by scanning densitometry.

**Trition Extraction**

Whole Cells—Harvested cells were resuspended in 0.5% (v/v) Tri-
ton X-100, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 50 mM 
Tris-HCl, pH 6.8 (Triton buffer), triturated and incubated for 10–15 
min at 25 °C (5, 31). Homogenates (0.5-1 mg of protein/ml, 100–2000 
μl sample volume) were then spun for 130,000 g-min in a microcen-
trifuge (15, 31). Aliquots from the Triton-soluble supernatants were 
resolved by SDS-PAGE as described above. Radiolabeled proteins 
were visualized by radioautography, and bands of interest were ana-
yzed by scanning densitometry.

**Isolated Fractions—Aliquots (125 μl) from fractions obtained from 
Percoll gradients were diluted 5-fold with Triton buffer, triturated, 
and incubated for 10-15 min at 25 °C. Aliquots were then spun for 
130,000 g-min in a microcentrifuge, and the supernatants were 
discarded. The pellets were resuspended in 100 μl of depolymerizing 
buffer, triturated, and incubated for 16 h at 4 °C. Proteins were then 
resolved by SDS-PAGE, and radioautograms were analyzed as 
described above.

**Vesicle Isolation**

Aliquots (75 μl) from fractions obtained from Percoll gradients 
were diluted to a total volume of 375 μl in sucrose buffer in the 
presence of 0.5% taurodeoxycholate. Aliquots were then 
incubated for 6 x 10^6 g-min, and the pellets were resuspended in 
100 μl of depolymerizing buffer, triturated, and incubated for 16 h at 
4 °C. Proportionate aliquots from the pellets (50 μl) and supernatants
(187 μl) were then analyzed by SDS-PAGE and radioautography as described above.

Miscellaneous Methods

Protein was determined using the method of Bradford (32) with human immunoglobulin as the protein standard. The Student's t test was used to compare the differences between experimental points. When indicated, results are presented as the mean ± S.D.

RESULTS

Subcellular Fractionation of Cultured Astrocytes—We have previously used 16% Percoll gradients and affinity labeling to examine the subcellular localization of 5′-D-II (5). After short-term (20 min) treatment of thyroid hormone-deficient astrocytes with 10 nM thyroxine, there is a redistribution of the affinity-labeled 29-kDa substrate-binding subunit of 5′-D-II (p29) within fractions that contain markers for the plasma membrane, endosomes, endoplasmic reticulum, and buoyant lysosomes. Because of the overlap of organelles in these fractions, we could not determine if this shift of 5′-D-II represents the transport of the affinity-labeled enzyme from the plasma membrane to another membrane pool.

In order to gain better resolution of organelles of similar density, the cytosolic fractions were discarded and fractions, from the 16% Percoll gradients with a buoyant density range of 1.084–1.056 g/ml that contain >90% of the membrane-bound organelles (3), were refractionated on 8% Percoll gradients. The distribution of marker enzymes after refractionation on 8% Percoll gradients is shown in Fig. 1. Na+K+ ATPase, a marker for the plasma membrane, is found predominantly in fractions 9 and 10 (density 1.041 g/dl), along with the major protein peak. The distribution of the β-subunit of the enzyme is broader than that of the catalytically active enzyme (found in a single peak in fraction 10), demonstrating the differential sensitivity between the assay for enzyme catalytic activity and the analysis of enzyme immunoreactivity. Actin is found predominantly in fractions 9 and 10 in the form of F-actin since all of the G-actin is found in the cytosolic fractions (5) and discarded prior to refractionation.

As seen in previous studies (5, 33), the lysosomal marker β-glucuronidase identifies two populations of lysosomes. The less dense population (fractions 8–11) consists of the buoyant lysosomes and contains ~38% of the enzymatic activity in the gradient, with the major peak in fraction 9. The dense lysosomes contain ~35% of the enzymatic activity and are found in fractions 13–18 (density 1.043–1.046 g/dl). The endoplasmic reticulum marker, glucose-6-phosphatase, also exhibits the bimodal distribution seen previously (5), with the major peak (fraction 11) of the less dense population migrating at a slightly higher density than the plasma membrane markers.

The endosomes were identified by following the internalized transferrin receptor (Fig. 1). Surface labeling of the transferrin receptor with [125I]transferrin at 4 °C resulted in ~70% of the radioactivity comigrating with the plasma membrane markers in fraction 9 and 10. After warming the cells to 37 °C for 10 min, ~20% of the [125I]transferrin is found in fractions 12 and 13 (density 1.042 g/dl). This represents internalization of the receptor-ligand complex to the endosomal pool, with ~55% of the [125I]transferrin remaining in the plasma membrane pool. A minor fraction (15–20%) of the radioactivity appears in fraction 22 and is unchanged by warming. It is unlikely that this minor population represents any specific intracellular organelle and, thus, represents dense aggregates of transferrin formed during refractionation.

Effect of Thyroxine on the Intracellular Transit of 5′-D-II—The greater separation of organelles achieved by successive gradient isolation allowed the effects of thyroid hormone on intracellular transit of 5′-D-II to be determined. Shown in Fig. 2A is the internalization pathway of 5′-D-II in cells treated with 10 nM thyroxine. As shown previously (5), 80–90% of the affinity-labeled substrate-binding subunit of 5′-D-II (p29) is initially found in fractions comigrating with the plasma membrane (fractions 9, 10), with ≤10% of the p29 found in higher density fractions.

Little movement of p29 between gradient fractions was initially detected after treatment with thyroxine, despite the marked decrease in 5′-D-II catalytic activity (Fig. 2A). Since ~60% of p29 becomes associated with F-actin within 20 min after the addition of thyroxine (5) and F-actin comigrates with the plasma membrane (Fig. 1), the lack of a dramatic shift in density of p29 coinciding with the loss of catalytic activity is anticipated. To establish that the formation of a p29-F-actin complex accounts for the lack of movement of p29 from the plasma membrane fractions after thyroxine treatment, organelles migrating in fraction 10 after 20-min treatment with thyroxine were diluted 5-fold and the cytoskeletal components separated from the membrane vesicles by low speed centrifugation at 130,000 g-min. Most of the p29 (444 ± 230 arbitrary units/fraction) was recovered in the cytoskeletal pellet along with the F-actin stress fibers. Depolymerization of the microfilaments present in fraction 10 prior to separating the membrane vesicles from the cytoskeleton resulted in the complete loss of p29 (<1 arbitrary units/fraction) from the cytoskeletal pellet.

In cells treated with thyroxine for 60 min, p29 shifts to a single fraction of higher density (fraction 12, density 1.042 g/dl (Fig. 2, A and B). Fraction 12 contains the peak of the marker for the endosomal pool (transferrin, Fig. 2B). This
endosomal-p29 pool is distinct from the plasma membrane, the two populations of lysosomes, and the major peak of the endoplasmic reticulum. There is no apparent transfer of p29 to the dense lysosomes after 60 min of thyroxine treatment. The small fraction of the total p29 (10–15%) spread over higher density fractions (fractions 15–21, Fig. 2A) remains stable in the presence of thyroxine. Incubation of thyroxine-treated astrocytes with either the lysosomotropic agents NH₄Cl or chloroquine (34) or the endocytosis inhibitor monodanycycladaverine (35) blocked the thyroxine-stimulated internalization of p29 (Table I).

**Intracellular Transit of p29 in Thyroid Hormone-deficient or T₃-treated Astrocytes**—Since dynamic regulation 5'D-II inactivation is an extranuclear action of thyroid hormone, the traditionally bioactive T₃ is >100-fold less potent than thyroxine (3–9), and 10 nM T₃ has no effect on 5'D-II inactivation over that seen in thyroid hormone-deficient conditions. Shown in Fig. 3 is the internalization pathway of 5'D-II in cells treated with 10 nM T₃. In contrast to the translocation of p29 solely to an endosomal pool in thyroxine-treated astrocytes, treatment of astrocytes with T₃ results in the translocation of p29 to the dense lysosomes. By 20 min, ~20% of the p29 is found in fractions 13–18, comigrating with the dense lysosomes. After 60 min, only 30% of the p29 remained in the plasma membrane fractions, while ~50% of the p29 is found in the dense lysosomes.

As shown in Table II, the loss of p29 from the plasma membrane fraction and subsequent accumulation in the lysosomes parallels the decrease in enzyme catalytic activity in cycloheximide-treated cells. By 60 min, the total cellular content of p29 decreased by 30–40% consistent with the degradation of this polypeptide in the lysosomes. The intracellular pathway of p29 under thyroid hormone-deficient conditions is identical to that seen in the T₃-treated cells (data not shown).

Treatment of thyroid hormone-deficient astrocytes with NH₄Cl, chloroquine, and monodanycycladaverine completely blocked accumulation of p29 in the dense fractions (Table I). Translocation of p29 from the plasma membrane fractions was blocked completely by NH₄Cl and partially by monodancycladaverine and chloroquine.  

**Role of the Actin Cytoskeleton in the Intracellular Transport of p29**—To examine the cytoskeletal-protein interactions involved in the intracellular movement of 5'D-II, we combined density gradient centrifugation with Triton X-100 extraction. In thyroid hormone-deficient astrocytes, >90% of the p29 is expressed as a percent of the total p29 in the gradient as determined by scanning densitometry; right panels, thyroid hormone-deficient, Bt-cAMP-stimulated cultured astrocytes were treated with 10 μM cycloheximide plus 10 nM thyroxine for varying times. Sonicates were assayed for 5'D-II activity as described under “Experimental Procedures.” Results are expressed as the percent of initial activity remaining. All points represent the mean of closely agreeing values in two or more experiments. B, subcellular localization of p29 after thyroxine-stimulated internalization. Fractions 8–16 were obtained after treatment with thyroxine for 60 min and were analyzed for p29 by autoradiography, the β-subunit of the Na⁺/K⁺ ATPase by Western analysis and glucose-6-phosphatase and β-glucuronidase by enzyme activity. The quantity of p29 and the β-subunit of the Na⁺/K⁺ ATPase are expressed as integrated optical density, in arbitrary units (au), per fraction. Glucose-6-phosphatase and β-glucuronidase enzyme activity is expressed the optical density at A₅₄₀ and A₅₄₅, respectively, in arbitrary units. The distribution of transferrin was determined in a separate experiment by labeling cultured astrocytes with [¹²⁵I]transferrin for 2 h at 4 °C, warming the cells to 37 °C for 5 min, then fractionating the cells as described above. Fractons 8–16 were then counted, and the data were expressed as cpm/fraction. Results represent the mean of duplicate values obtained in two experiments.
Thoracic and suprarenal simulation studies in vivo by a model in which p29 is isolated from the plasma membrane and the lysosomes by separation through Percoll gradients. The half-life of p29 is determined by measuring the integrated optical density of p29 in the plasma membrane and the lysosomes at different times after the addition of thyroid hormone-deficient conditions, or in the presence of 10 nM T3 (data not shown).

The binding of p29 to F-actin was examined directly by isolating the p29-containing fractions on Percoll gradients, extracting the isolated fractions with Triton X-100 and then examining the amount of p29 in the insoluble F-actin pellets after centrifugation. As shown in Fig. 5, the quantity of p29 found in the F-actin pellet increases ~3-fold after 20 min of thyroxine treatment. With longer times, the p29 content in the F-actin pellet decreases back to initial levels, again coinciding with the shift of p29 to an endosomal pool.

Demonstration That p29 Is Internalized as Part of a Vesicle in Thyroxine-Treated Astrocytes—Whether the p29 subunit of 5'D-II is translocated from the plasma membrane to the endosomes as an isolated subunit or as part of a vesicle-associated holoenzyme was then examined. The ability of lysosomotropic agents and endocytosis inhibitors to block the internalization of p29 is consistent with the idea that p29 is internalized in association with a membrane vesicle. As shown in Fig. 6, all of the p29 transferred to the endosomal pool by 60 min after treatment with thyroxine is found in the high speed membrane pellet after centrifugation in the absence of detergent. Addition of 0.5% taurodeoxycholate to the endosomal fraction prior to centrifugation solubilized 65.6% of the

![Image](image_url)
**FIG. 4. Effect of thyroxine on the Triton-solubility of p29.** Serum-free, Bt$_2$AMP-stimulated astrocytes were affinity-labeled then treated with 10 nM thyroxine for varying times, and the Triton-soluble fraction of the whole cells isolated as described under "Experimental Procedures." The quantity of p29 in the Triton-soluble fractions was determined by scanning densitometry of autoradiograms after SDS-PAGE. Results are presented as a percent of control which is defined as the quantity of p29 prior to the addition of thyroxine and was 8.0 ± 6.5 arbitrary units/mg protein in these studies. Results are the mean of triplicate determinations in at least three experiments. *, p < 0.05 as compared to values obtained at 0 min.

**FIG. 5. Time course of the binding of p29 to F-actin in thyroxine-treated cells.** Thyroid hormone-deficient, Bt$_2$AMP-stimulated cultured astrocytes were affinity-labeled, treated with 10 nM thyroxine, collected, and fractionated through 16 and then 8% Percoll gradients as described under "Experimental Procedures." The pellets were resuspended in 100 μl of depolymerizing buffer, incubated for 16 h at 4°C, and analyzed for p29 by radioautography after SDS-PAGE. Results are the mean of four to six closely agreeing values obtained in two experiments and are presented as integrated optical density, in arbitrary units (au), of the total p29 in the pellets. *, p < 0.05 as compared to values obtained at 0 min.

p29, indicating that p29 is internalized as part of a membrane-bound vesicle.

**DISCUSSION**

Transport of proteins, lipids, and organelles within cells is a fundamental cellular process that is poorly understood. The individual intracellular components of the endocytotic pathway have been extensively characterized, but the locomotive force that translocates and targets the internalized vesicles to their eventual location within the cells remains obscure. Recent studies suggest that the cytoskeleton may be a key component in the intracellular transport of a variety of organelles and vesicles. In particular, disruption of the cytoskeletal elements has been shown to disrupt the endocytotic pathway in macrophages (13, 14), rat Kupffer cells (18), rat hepatocytes (17), and 3T3 cells (19, 20). Most studies on the mechanisms of cytoskeletal-mediated vesicle transport have focused on the role of the microtubules and their associated motor proteins, as the microtubules have been shown to be major determinants of tubular lysosome morphology (13, 14) and are responsible for the internalization of the acetylcholine receptor (36) and the asialo-glycoprotein receptor (17).

Our laboratory has focused on the microfilaments as the key component in the intracellular transport of the short-lived integral membrane protein, type II iodothyronine 5'-deiodinase. This enzyme is highly regulated by thyroxine, which acts to markedly increase the turnover of 5'D-II (3-9). The actions of thyroxine on the turnover of 5'D-II are blocked by chemical disruption of the microfilaments with cytochalasins (5, 6). Subsequent studies have shown that thyroxine stimulates actin polymerization (7) and promotes the binding

[Figures 4 and 5 are not transcribed but are referenced.]
of 5'D-II to F-actin, which leads to internalization of an F-actin-5'D-II complex (5). Disruption of the microtubules with colchicine has no effect on the inactivation/internalization of 5'D-II in the presence or absence of thyroid hormone. Thus, the hormonally regulated turnover of type II iodothyronine 5'-deiodinase is an excellent model in which to study the role of the cytoskeleton, specifically the microfilaments, in intracellular protein trafficking.

The present study was designed to examine where in the cell 5'D-II is transported to and the effect of thyroxine and, hence, the role of the microfilaments, on the route of internalization of 5'D-II. We have shown that 5'D-II is internalized via both microfilament-dependent and microfilament-independent mechanisms and that the targeting of 5'D-II to a particular endocytic pathway is regulated by thyroxine (Fig. 7). In the presence of thyroxine, 5'D-II binds to F-actin and the microfilaments translocate the enzyme from the plasma membrane to the endosomes. Following transport to the endosomes, 5'D-II is uncoupled from the microfilaments. Interestingly, the enzyme remains in the endosomal pool for extended periods, as further transport of 5'D-II to the dense lysosomes was not observed in the time frames examined.

5'D-II is also internalized into an endosomal pool via a microfilament-independent mechanism in the absence of thyroxine. However, in contrast to the thyroxine-treated pathway, the enzyme is rapidly transferred from the endosomes to the dense lysosomes, preventing any accumulation of 5'D-II in the endosomes. Whether the endosomal population 5'D-II that is cycled through in the absence of thyroxine is the same endosomal pool that 5'D-II is transported to by the microfilaments in the presence of thyroxine is uncertain.

Previously, functions attributed to the actin cytoskeleton have been limited to the regional distribution of proteins and surface receptors within the plasma membrane (36-40) and global changes in plasma membrane organization following acid stimulation in gastric parietal cells (41). However, disruption of the microfilaments has also been shown to block transport of asialo-glycoproteins (17) and labeled phagosomes (18) from the endosomes to the lysosomes. The present study now demonstrates a primary role of the microfilaments in the endocytosis of the short-lived membrane protein, 5'D-II. In addition, since thyroxine regulates the presence of the F-actin stress fibers by modulating actin polymerization (7), these data demonstrate that the stress fibers are responsible for the hormonally regulated endocytosis of 5'D-II.

While we have shown that thyroxine stimulates actin polymerization and targets 5'D-II to a microfilament-dependent endocytotic pathway, the identity of the targeting signal regulated by thyroxine remains unclear. Because the actions of thyroxine on the microfilaments that result in differential pathways of endocytosis of 5'D-II occur independently of interactions with the nuclear thyroid hormone receptor (6, 7), other sites of action must be considered. The ability of thyroxine to promote actin polymerization alone is insufficient to explain the activation of the microfilament-mediated pathway of endocytosis since restoration of the stress fibers in thyroid hormone-deficient astrocytes with retinoic acid does not increase inactivation/internalization of 5'D-II (42). Likewise, deiodination of thyroxine is not the targeting signal, as covalent modification of the active site of the substrate-binding subunit of 5'D-II with the affinity label, BrAcT4, fails to alter the thyroxine-dependent internalization of the enzyme.

Although high affinity binding sites for thyroid hormone have been shown to be present in the erythrocyte plasma membrane (43), cell surface receptors for thyroxine have not been described in astrocytes. However, the demonstration that 5'D-II is internalized as part of a vesicle raises the possibility that thyroxine may be interacting with other membrane proteins that act as chaperons to bind the enzyme to F-actin during the microfilament-mediated internalization. These as yet unidentified proteins may play a key role in intracellular targeting. Implicit in this hypothesis is that potential chaperon proteins are constitutively expressed, as the effects of thyroxine on endocytosis of 5'D-II occurs in the absence of new proteins synthesis.

The ultimate fate of the 5'D-II transported to the endosomes by the microfilaments in the presence of thyroxine is unknown. We were unable to detect further transport of the endosomal 5'D-II to other cellular compartments in the time frames examined. Preliminary studies have failed to show evidence of recycling of 5'D-II back to the plasma membrane (44). Further studies are necessary to determine the metabolic fate of this enzyme.

In summary, we have shown that several endocytic pathways exist within astrocytes and that targeting to a specific pathway is hormonally regulated. Thyroxine targets 5'D-II to the endosomes by activating enzyme-F-actin interactions important in microfilament-dependent intracellular protein trafficking.

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Fig. 7. Endocytosis pathways of 5'D-II in astrocytes. Diagrammatic representation of the effects of thyroid hormone on the endocytosis of 5'D-II. In the absence of thyroid hormone, or in the presence of T₄, 5'D-II is internalized from the cytoplasmic surface of the plasma membrane (PM) by endocytosis and transported to the dense lysosomes, preventing any accumulation of 5'D-II. In the presence of thyroxine (T₄), 5'D-II binds to the F-actin stress fibers and is transported to the endosomes.
Internalization Pathways of 5'D-II

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