Degradation and Recycling of the Substrate-binding Subunit of Type II Iodothyronine 5′-Deiodinase in Astrocytes*

(Received for publication, November 16, 1995, and in revised form, March 1, 1996)

Alan P. Farwell‡, Marjorie Safran, Susan Dubord, and Jack L. Leonard

From the Molecular Endocrinology Laboratory, Departments of Medicine, Nuclear Medicine, and Molecular and Cellular Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Thyroxine dynamically regulates levels of type II iodothyronine 5′-deiodinase (5′-D-II) by modulating enzyme inactivation and targeting the enzyme to different pathways of internalization. 5′-D-II is an ~200-kDa multimeric protein containing a 29-kDa substrate-binding subunit (p29) and an unknown number of other subunits. In the absence of thyroxine (T4), p29 is slowly endocytosed and transported to the lysosomes. T4 treatment rapidly activates an actin-mediated endocytotic pathway and targets the enzyme to the endosomes. In this study, we have characterized the influence of T4 on the intracellular trafficking of 5′-D-II. We show that T4 accelerates the rate of 5′-D-II inactivation by translocating the enzyme to the interior of the cell and by sequestering p29 in the endosomal pool without accelerating the rate of degradation of p29. This dichotomy between the rapid inactivation of catalytic activity and the much slower degradation of p29 is consistent with the reuse of p29 in the production of 5′-D-II activity. Immunocytochemical analysis with a specific anti-p29 IgG shows that pulse affinity-labeled p29 reappears on the plasma membrane ~2 h after enzyme internalization in the presence of T4, indicating that p29 is recycled. Despite the ability of p29 to be recycled in the T4-treated cell, 5′-D-II catalytic activity requires ongoing protein synthesis, presumably of another enzyme component(s) or an accessory enzyme-related protein. In the absence of T4, enzyme inactivation and p29 degradation are temporally linked, and pulse affinity-labeled p29 is internalized and sequestered in discrete intracellular pools. These data suggest that T4 regulates fundamental processes involved with the turnover of integral membrane proteins and participates in regulating the inter-relationships between the degradation, recycling, and synthetic pathways.

Type II iodothyronine 5′-deiodinase (5′-D-II)1 is a multimeric integral membrane protein (~200 kDa) that catalyzes T4 to T3 conversion in the brain (1–4). Thyroid hormone, specifically T4, accelerates the rate of degradation of p29 in the production of 5′-D-II (1, 5–8). D-II remains uncertain in the presence of T4. The 29-kDa substrate-binding subunit (p29) of 5′-D-II is covalently modified by the alkylating affinity label N-bromoacetyl-L-thyroxine (BrAcT4), allowing the enzyme to be identified without measuring catalytic activity (10). Utilizing cultured astrocytes that retain all of the 5′-D-II regulatory aspects seen in the brain in vivo and that express high levels of 5′-D-II activity in the presence of cyclic AMP (11), we have previously shown that T4 dynamically regulates 5′-D-II levels by directing the enzyme to use different pathways of internalization (8). Under T4-deficient conditions, p29 is internalized via the traditional endocytotic pathway and is slowly transported through the endosomes to the lysosomes. In contrast, T4 treatment activates specific protein-F-actin interactions involved in actin-mediated endocytosis and targets the enzyme to the endosomes, where the internalized 5′-D-II-containing vesicle remains without subsequent transit to the lysosomes. Recycling of the catalytically active enzyme back to the plasma membrane has not been observed in the short time frames examined previously; thus, the fate of the endosomal pool of internalized 5′-D-II remains uncertain in the presence of T4. The endosomes are a collection of vesicles located in the perinuclear space (12, 13). Vesicles internalized via endocytosis initially exist as early endosomes and contain a mixture of polypeptides with differing fates. Vesicles containing proteins to be recycled, such as the transferrin and insulin receptors, are directed back to the plasma membrane (14), while vesicles containing proteins destined for degradation evolve into late endosomes and are targeted to the lysosomes. In the secretory pathway, vesicles containing newly synthesized membrane proteins pass from the endoplasmic reticulum to the Golgi stack before exiting through the trans-Golgi network, where they are targeted to the plasma membrane directly or routed through the endosomes (15, 16). From the endosomes, the secretory vesicles may be directed to the plasma membrane, to the lysosomes, or to vesicle storage pools. The sorting mechanism that determines the destination of the differing vesicles that compose the endosomes is still unclear (17).

The T4-dependent regulation of the pathways of 5′-D-II inactivation/internalization suggests a role for hormonal regulation in vesicle-mediated protein transport (8). In this study, we show that 1) the rate of degradation of the 29-kDa substrate-
binding subunit of 5'D-II (p29) is unaffected by T₄; 2) in the presence of T₃, p29 is recycled back to its site of action on the plasma membrane; and 3) despite recycling of p29, 5'D-II catalytic activity requires de novo synthesis of additional enzyme components. Since T₃ determines whether p29 is recycled back to the plasma membrane or is routed to the lysosomes, these data suggest that, in addition to regulating the internalization pathway of 5'D-I, T₃ participates in regulating the inter-relationships between the degradation, recycling, and synthetic pathways.

EXPERIMENTAL PROCEDURES

Materials—Pregnant (16–17-day gestation) rats were obtained from Charles River Laboratories (Kingston, NY). T₄ and BSA was purchased from Sigma. T₃ was obtained from Henning GmbH, and dihydroxytocalasin B was obtained from Calbiochem. Dulbecco's modified Eagle's medium (DMEM), antibiotics, Hanks's solution, and 0.25% trypsin were obtained from Life Technologies, Inc., and defined bovine calf serum (heat-inactivated) was from Hydnone Laboratories. Culture flasks were obtained from Nunc, and 24-well tissue culture plates were obtained from Falcon All other reagents used were of the highest purity commercially available.

Cell Culture—Rat type I astrocyte cultures were obtained by enzymatic dispersion of neonatal rat brains as described previously (18). Cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C in DMEM supplemented with 15 mM sodium bicarbonate, 33 mM glucose, 1 mM sodium pyruvate, and 15 mM HEPES, pH 7.4, with 10% (v/v) calf serum, 50 units/ml penicillin, and 90 μg/ml streptomycin. The culture medium was changed three times weekly, and cells were subcultured (2–3 × 10⁴ cells/cm²) when they reached confluence (7–10 days). Confluent cells from passages 2–4, containing >95% astrocytes as determined by staining for the astrocyte-specific protein, glial fibrillary acidic protein (19), were utilized for experiments.

Induction and Measurement of 5'D-I Activity—Steady-state levels of 5'D-I were induced by incubating the cells for 6 h in a defined medium containing 75 mM DMEM and 0.1% BSA + thyrotropin (TSH) or thyroxine (T₄), followed by a 16-h stimulation with 1 μM Bt₂CAMP and 100 μM hydrocortisone. Cells were harvested by scraping in ice-cold 8 mM sodium phosphate buffer, pH 7.4, containing 2.7 mM KCl and 137 mM NaCl and collected by centrifugation. 5'D-I activity was determined in cell sonicates by the iodide release method at 2 μM T₃ and 20 μM diithiothreitol in the presence of 1 μM propylthiouracil. Units are expressed as femtomoles of 131I released per hour.

The turnover of 5'D-II was determined by assaying enzyme activity 5–60 min after inhibition of protein synthesis with 100 μM cycloheximide in 50 mM HEPES and 1 mg/ml BSA in buffered Hanks' solution, pH 7.0, in the presence or absence of 10 μM T₄. The turnover of 5'D-II in the presence of BFA was determined by preincubating cells with 5 μg/ml BFA for 15 min before the addition of 100 μM cycloheximide. Cells were then collected as described above and assayed for 5'D-II activity.

Degradation of p29—The p29 polypeptide was affinity-labeled in confluent astrocytes expressing steady-state levels of 5'D-II in the absence of thyroid hormone by incubation for 20 min with either 0.4 or 10 μM BrAc[125I]T₄, 1 μM diithiothreitol, and 50 mM HEPES in buffered Hanks' solution, pH 7.0. The labeling medium was removed, and the cells were washed free of unbound affinity label and then chased for 30–120 min with either 10 μM T₄ or T₃ or with no hormone in 50 mM HEPES and 1 mg/ml BSA in buffered Hanks' solution, pH 7.0. Cells were collected and sonicated. Affinity-labeled proteins were identified after SDS-polyacrylamide gel electrophoresis and autoradiography. The quantity of p29 was determined by scanning densitometry.

Immunocytochemistry—Cells were seeded onto glass coverslips (22 × 22 mm) coated with poly-l-lysine (10 μg/ml) and grown for 24 h. 5'D-II activity was induced as described above. Cells were affinity-labeled for 20 min with 10 μM BrAc[125I]T₄ in buffered Hanks' solution containing 10 μM diithiothreitol and 50 mM HEPES, pH 7.0, and washed and chased for 0–90 min in DMEM with 1 μg/ml BSA, 100 μM hydrocortisone in the presence and absence of 10 μM T₄. All cells were treated with 10 μM colchicine to relax the cell borders for 60 min prior to fixation. Cells were then collected in iced 4% paraformaldehyde and permeabilized with ice-cold methanol. 5'D-IIwas identified by incubation with an antiserum raised against p29 (see accompanying paper (31)) or with an anti-T₄ IgG that recognizes the BrAcT₄ moiety, and immune complexes were identified by incubation with Texas Red-conjugated antirabbit IgG. Coverslips were then mounted and examined by laser scanning confocal microscopy with a depth of resolution of 0.5 μm.

FIG. 1. Acute effects of thyroid hormone on the turnover of p29 and 5'D-II catalytic activity in astrocytes. Steady-state levels of 5'D-II activity were induced in confluent astrocytes in the absence of thyroid hormone as described under "Experimental Procedures." A, p29 turnover. Hormone-free cells were affinity-labeled and then chased with 10 nM T₃, 10 nM T₄, or no hormone for 30–90 min. Results are expressed as a percent of the starting p29 content (optical density/mg of protein) and represent the means of duplicate values in at least three experiments. B, turnover of 5'D-II catalytic activity. 5'D-II activity was determined after treatment of hormone-free cells with 10 nM T₄, 10 nM T₃, or no hormone for 5–60 min in the presence of 100 μM cycloheximide. Results are expressed as a percent of the starting 5'D-II activity in the absence of thyroid hormone (2177 fmol of 131I released per mg of protein/h) and represent the means of triplicate values in at least three experiments. T₄-treated, cells incubated in the presence of 10 nM T₃; T₃-treated, cells incubated in the presence of 10 nM T₃; TH-Deficient and T₄-Deficient, cells incubated in the absence of T₃ and T₄.

Statistical Analysis—Where indicated, results were analyzed by unpaired Student's t test.

RESULTS

Degradation of p29—The 29-kDa substrate-binding subunit of 5'D-II (p29) is selectively affinity-labeled by BrAcT₄ (4, 10). In the absence of T₄, p29 is internalized and targeted to the lysosomes, whereas in the presence of T₄, p29 remains indefinitely in the endosomal pool localized in the perinuclear space of the cell (7, 8). In light of the different metabolic fates of proteins residing in these two intracellular compartments, we examined the effect of thyroid hormone on the degradation of p29. Cells were grown in thyroid hormone-free medium and stimulated with Bt₂CAMP and hydrocortisone to express high levels of 5'D-II (11). P29 was affinity-labeled, and cells were then chased with either 10 nM T₄ or T₃ or with no hormone for up to 90 min. The cellular content of p29 and the levels of catalytically active 5'D-II were analyzed at 0–90 min after the addition of hormone. As shown in Fig. 1A, the disappearance of p29 over time was unaffected by thyroid hormone, while >80% of the catalytic activity of 5'D-II was lost due to the presence of T₄ (Fig. 1B). Little or no other smaller affinity-labeled degradation products from the affinity-labeled p29 protein were observed over this time frame (data not shown).

Shown in Table I are the steady-state levels of p29 in cells grown in the presence and absence of T₄. As reported previ-
Degradation and Recycling of 5’D-II

Effect of thyroid hormone on the half-life of p29 and 5’D-II in astrocytes

Confluent cells were incubated for 8 h in defined medium containing 10 nM T4 or no hormone and then stimulated for 16 h with Bt2cAMP and hydrocortisone. Turnover was then determined as described under “Experimental Procedures.”

| p29 content | 10 nM T4 | Thyroid hormone-deficient |
|-------------|----------|--------------------------|
| Steady-state levels (OD/mg protein) | 17.7 | 35.1 |
| $t_\frac{1}{2}$ (h$^{-1}$) | 1.7 | 1.5 |
| Appearance rate (h$^{-1}$) | 7.3 | 16.2 |

5’D-II activity

| Steady-state levels (units/mg protein) | 200 | 2177 |
| $t_\frac{1}{2}$ (h$^{-1}$) | 0.2 | 2.2 |
| Production rate (h$^{-1}$) | 692 | 718 |

As shown previously (6, 10), the production rate of differences between the effects of T4 on the respective cycloheximide for up to 4 h, and 5’D-II in the presence of continued presence of 10 nM T4, and 5’D-II activity was assayed after increasing periods of time. Results represent the means of triplicate values in at least three experiments. Units are femtomoles of 1⁄2 released per hour. Control, T4-replete cells; CHX, T4-replete cells treated with cycloheximide; CHX/BFA, T4-replete cells treated with cycloheximide plus brefeldin A.

Effect of cycloheximide and brefeldin A on 5’D-II activity in T4-treated astrocytes. Confluent cells were incubated for 8 h in defined medium containing 10 nM T4 and stimulated for 16 h with Bt2cAMP and hydrocortisone. Cells were then treated with 100 μM cycloheximide in the presence and absence of brefeldin A (5 μg/ml) while in the continued presence of 10 nM T4, and 5’D-II activity was assayed after increasing periods of time. Results represent the means of triplicate values in at least three experiments. Units are femtomoles of 1⁄2 released per hour. Control, T4-replete cells; CHX, T4-replete cells treated with cycloheximide; CHX/BFA, T4-replete cells treated with cycloheximide plus brefeldin A.

Effect of Brefeldin A on 5’D-II Catalytic Activity—The differences between the effects of T4 on the respective $t_\frac{1}{2}$ values of p29 and 5’D-II catalytic activity, our observation that p29 accumulates in the endosomal pool in the presence of T4 (8), and the observation that p29 is found in the endosomal pool in catalytically inactive astrocytes (see accompanying paper (31)) raise the possibility that this multimeric enzyme is recycled. To examine this possibility, recycling of 5’D-II activity was determined in protein synthesis-blocked cells kept for extended periods of time. Cells expressing 5’D-II activity in T4-replete medium were incubated in the presence and absence of 100 μM cycloheximide for up to 4 h, and 5’D-II activity was assayed. As shown in Fig. 2, 5’D-II activity fell to undetectable levels in cycloheximide-blocked cells, with little or no recovery of catalytic activity observed at any time point. Next, we examined the effects of BFA on steady-state levels of 5’D-II in the presence of continued protein synthesis (Fig. 3). BFA had no effect on steady-state levels in T4-replete cells during treatment periods up to 60 min. In contrast, BFA treatment led to a steady increase in 5’D-II activity in T4-deficient cells, resulting in a doubling of enzyme activity by 60 min. Analysis of the disappearance kinetics of 5’D-II revealed that BFA prolonged the biological half-life of the catalytically active enzyme in both T4-deficient (Fig. 4A) and T4-replete (Fig. 4B) astrocytes. Since the biological half-life of 5’D-II activity in T4-replete cells was prolonged in the presence of BFA and the levels of enzyme remained unchanged, BFA treatment must cause a proportional fall in enzyme production. 5’D-II generation in T4-deficient cells was minimally affected by BFA, as there was a concordant increase in both $t_\frac{1}{2}$ and enzyme levels. These data imply that the sources of the catalytically active holoenzyme are different in cells grown in the presence and absence of T4.

One explanation for these differential effects of BFA on 5’D-II activity is that one or more of the enzyme-associated polypeptides are recycled in cells grown in the presence of T4. Thus, in T4-treated cells where 5’D-II is rapidly internalized to the endosomes (7, 8), BFA treatment should prevent the recycling of enzyme polypeptide(s) from joining the de novo enzyme production pathway, leading to an accumulation of inactive enzyme precursors in the fused vesicle pool and thereby decreasing the observed enzyme production rate. To test this hypothesis, we determined the effects of BFA on the accumulation of catalytically active 5’D-II after depolymerization of the F-actin cytoskeleton in T4-replete astrocytes. Depolymerization of F-actin by dihydrocytochalasin B blocks T4-mediated endocytosis and results in the accumulation of 5’D-II activity at a rate equal to the production rate of enzyme catalytic activity (6). An accelerated rate of accumulation of catalytic activity is expected if a pool of enzyme precursors is formed in the presence of BFA.

As shown in Fig. 5, the addition of dihydrocytochalasin B to T4-replete astrocytes caused the steady accumulation of 5’D-II activity as reported previously (6). Pretreating the T4-replete cells with BFA increased the initial rate of enzyme accumula-
Fig. 3. Time course of the effect of brefeldin A on 5'D-I1 activity in astrocytes. Steady-state levels of 5'D-I1 activity were induced in confluent astrocytes as described under "Experimental Procedures." BFA (5 μg/ml) was then added for 15-60 min, and 5'D-I1 activity was assayed. Results are expressed as a percent of the starting 5'D-I1 activity (femtomoles of I1 released per milligram of protein/hour) and represent the means of triplicate values in at least three experiments. T4-Deficient, cells incubated in the absence of T4; T4-Treated, cells incubated in the presence of 10 nM T4. * p < 0.0005 compared with the T4-replete cells.

Fig. 4. Effect of brefeldin A on the turnover of 5'D-I1 activity in astrocytes. Steady-state levels of 5'D-I1 activity were induced in confluent astrocytes incubated in either the presence or absence of 10 nM T4 as described under "Experimental Procedures." Cells were incubated in the presence (●) or absence (○) of BFA (5 μg/ml) for 15 min, and then protein synthesis was blocked by the addition of 100 μM cycloheximide. 5'D-I1 activity was assayed at increasing times after the addition of cycloheximide. Results are expressed as a percent of the starting 5'D-I1 activity and represent the means of triplicate values in at least two experiments. A, cells incubated in the absence of thyroid hormone; B, cells incubated in the presence of 10 nM T4.

Fig. 5. Effect of dihydrocytochalasin B on 5'D-I1 activity in T4-replete astrocytes pretreated with brefeldin A. Steady-state levels of 5'D-I1 activity were induced in confluent astrocytes in the presence of 10 nM T4 as described under "Experimental Procedures." Cells were incubated with BFA (5 μg/ml) for 0, 30, or 60 min prior to the addition of 10 μM dihydrocytochalasin B. 5'D-I1 activity was assayed at 10, 20, and 30 min after the addition of dihydrocytochalasin B. Results are expressed as a percent of the initial 5'D-I1 activity and represent the means of triplicate values in at least three experiments.

Degradation and Recycling of 5'D-I1 in an intracellular pool.

Immunocytochemical Analysis of the Intracellular Transit of the Substrate-binding Subunit of 5'D-I1—Direct examination of the intracellular transit of the enzyme polypeptide(s) was performed by using a specific IgG directed against the affinity-labeled 29-kDa substrate-binding subunit of 5'D-I1 (anti-p29 IgG) (see accompanying paper (31)). T4-deficient astrocytes were grown on glass coverslips, pulse-affinity-labeled with BrAcT4, and then chased with either 10 nM T4 or no hormone. Cells were fixed after increasing periods of time, stained with anti-p29 IgG, and examined by laser scanning confocal microscopy.

As shown in Fig. 6A (arrows), punctate staining is present in a "rim" pattern at the periphery of the cell in the thyroid hormone-deficient, affinity-labeled astrocyte, while the interior of the cell is largely devoid of immunoreactive staining. This rim pattern is consistent with a plasma membrane location for the staining. After a 20-min treatment with T4, the majority of the staining is located in the perinuclear space within the cell (Fig. 6B, P), with little immunoreactive staining remaining on the plasma membrane (arrows). These patterns are consistent with previous studies done with an anti-T4 IgG that show that affinity-labeled polypeptide(s), predominantly p29, are located on the cytoplasmic leaflet of the plasma membrane in the thyroid hormone-deficient cell and are translocated to the perinuclear space within 20 min of exposure to T4 (7). Similarly, previous studies have shown that the majority of affinity-labeled p29 after short-term exposure (20 min) to T4 resides in the endosomal pool (8). After a 2-h exposure to T4, prominent punctate staining is again found at the periphery of the cell in a rim pattern (Fig. 6C, white arrows), along with clusters of staining present diffusely throughout the cell (black arrow). Since anti-p29 IgG selectively recognizes affinity-labeled p29 (see accompanying paper (31)), these data show that pulse-labeled p29 was internalized and recycled back to the plasma membrane in T4-treated astrocytes. In contrast to the T4-treated cells, immunoreactive staining in the T4-deficient cell is clustered into discrete regions within the cell (Fig. 6D, black arrow), with little staining remaining on the plasma membrane (white arrows). Previous studies have shown that affinity-labeled p29 is transported from the plasma membrane to discrete intracellular pools identified as lysosomes in T4-deficient cells (8).

The effects of BFA on the recycling of pulse-labeled p29 are shown in Fig. 7. Pulse-labeled p29 was identified with an anti-T4 IgG that recognizes the BrAcT4-labeled enzyme as described previously (7). Punctate staining in a rim pattern is present in the cells treated with T4 for 2 h, indicating a plasma
The cell and rim staining is not present (Fig. 7, predominantly clustered within the cell at 2 h, with little effect on the nucleus. Shown are representative images obtained from 30–40 images per condition. A, thyroid hormone-deficient, affinity-labeled cells, no chase period; B, thyroid hormone-deficient, affinity-labeled cells treated with T$_4$ for 20 min; C, thyroid hormone-deficient, affinity-labeled cells treated with T$_4$ for 2 h; D, thyroid hormone-deficient, affinity-labeled cells incubated for 2 h without hormone. N, nucleus; P, perinuclear space. Marker bars = 10 µm.

**Fig. 6. Immunocytochemistry of the intracellular transit of 5′D-II.** Astrocytes were grown on coverslips, and steady-state levels of 5′D-II activity were induced as described under “Experimental Procedures.” Cells were affinity-labeled with 10 nm BrAcrT$_4$ for 20 min and then chased with either 10 nm T$_4$ or no hormone for up to 2 h. Cells were fixed and stained with an anti-p29 IgG, and immune complexes were visualized by indirect immunofluorescence. Images were obtained by confocal microscopy at the level of the nucleus. Shown are representative images obtained from 30–40 images per condition. A, thyroid hormone-deficient, affinity-labeled cells, no chase period; B, thyroid hormone-deficient, affinity-labeled cells treated with T$_4$ for 20 min; C, thyroid hormone-deficient, affinity-labeled cells treated with T$_4$ for 2 h; D, thyroid hormone-deficient, affinity-labeled cells incubated for 2 h without hormone. N, nucleus; P, perinuclear space. Marker bars = 10 µm.

**Fig. 7. Effect of brefeldin A on the intracellular transit of 5′D-II.** Astrocytes were grown on coverslips, and steady-state levels of 5′D-II activity were induced as described under “Experimental Procedures.” Cells were affinity-labeled with 10 nm BrAcrT$_4$ for 20 min and then chased for 2 h in the presence (right panels) and absence (left panels) of BFA (5 µg/ml). Cells were fixed and stained with an anti-T$_4$ IgG, and immune complexes were visualized by indirect immunofluorescence. Images were obtained by confocal microscopy at the level of the nucleus. Marker bar = 10 µm. Upper panels, cells chased in serum-free medium with 10 nm T$_4$; lower panels, cells chased in serum-free (SF) medium without hormone.

In this study, we have characterized the intracellular trafficking of 5′D-II in cAMP-stimulated astrocytes and have shown that T$_4$ regulates fundamental processes involved in the turnover of integral membrane proteins. Specifically, T$_4$ shuttles plasma membrane proteins to the endosomes by switching from an actin-independent to an actin-mediated internalization pathway. Once in the endosomes, T$_4$ modulates the sorting mechanism to select recycling pathways over degradation pathways. Thus, T$_4$ participates in regulating the inter-relationships between the degradation, recycling, and synthetic pathways.

The evidence the T$_4$ shifts 5′D-II to a recycling pathway is 3-fold. First, there is a dichotomy between the rapid inactivation of catalytic activity and the much slower degradation of the 29-kDa substrate-binding subunit (p29) in euthyroid cells, consistent with the reuse of this protein in the production of catalytically active 5′D-II. Second, there is an increased rate of accumulation of 5′D-II activity in brefeldin A-treated euthyroid cells after depolymerization of the F-actin microfilaments, consistent with the storage of enzyme-related polypeptides in an intracellular pool that is available to the synthetic pathway. Finally, using anti-p29 antisera, pulse-labeled p29 reappears on the plasma membrane ~2 h after internalization in the presence of T$_4$.

In contrast, the 5′D-II-containing vesicles in T$_4$-deficient cells are sorted through the endosomes to the degradation pathway, ending up in the lysosomes (8). Enzyme inactivation parallels p29 degradation in the absence of T$_4$. Interestingly, the degradation rate of p29 is similar whether it is sorted directly to the lysosomes in the absence of T$_4$ or is routed through one or more recycling sequences in the presence of T$_4$.

Since 5′D-II is a multimeric enzyme, there are two possibilities for recycling of this subunit. First, the holoenzyme may remain intact waiting for the synthesis of an accessory protein that either targets the enzyme to the plasma membrane or activates catalytic activity once it arrives at the plasma membrane. Consistent with this hypothesis, we showed that p29 in catalytically inactive astrocytes is part of a 180–200-kDa complex found in the endosomes (see accompanying paper (31)). Cyclic AMP induces the transcription of a 5′D-II “activating factor,” leading to the translocation of 5′D-II to the plasma membrane, activation of catalytic activity, and an increase in the apparent molecular mass of 5′D-II from 200–220-kDa range. Alternatively, the holoenzyme may be disassembled, with one or more subunits shuttled to the lysosomes and p29 combining with other newly synthesized subunits. At least one component of the catalytically active enzyme requires de novo synthesis since ongoing protein synthesis is required to maintain catalytic activity. It is likely that this latter component is the cAMP-inducible activating factor.

**DISCUSSION**

Previous work on the T$_4$-dependent regulation of 5′D-II activity identified the inactivation/internalization pathway as the primary site of action of T$_4$ (6–8, 23, 24). Indeed, when measuring catalytic activity, T$_4$ markedly increases the rate of inactivation of enzyme activity without affecting the production rate of 5′D-II activity (6). However, analysis of the effects of T$_4$ on the turnover of the p29 subunit revealed a more complex production pathway for catalytically active 5′D-II. Brefeldin A, a fungal toxin that disconnects the secretory, recycling, and degradation pathways for membrane proteins, decreased both the production rate and inactivation rate of the catalytically active enzyme in euthyroid cells, but had little effect on the production rate in hypothyroid cells. These data
Degradation and Recycling of 5’D-II

**Fig. 8. Model of the intracellular transit of 5’D-II in astrocytes.**

A, proposed intracellular transit of 5’D-II in the presence of T₄; B, hypothetical activating factor; ER, endoplasmic reticulum; TGN, trans-Golgi network.

The production of catalytically active 5’D-II differs in the presence and absence of T₄.

One possible explanation for this dichotomy is that the production of 5’D-II in the presence of T₄ uses both de novo synthesis and preformed subunits recycled from the endosomes. In euthyroid astrocytes, we found that BFA prevented the return of 5’D-II to the plasma membrane and led to an accumulation of 5’D-II polyepitope(s) in intracellular membrane pools (Fig. 7). In contrast, in hypothyroid cells, all components of catalytically active 5’D-II are produced de novo, and BFA did not affect the production rate. Since at least one component of catalytically active 5’D-II needs to be newly synthesized in both cases, it appears that fusion of vesicles from the Golgi and endosomal pool as well as de novo synthesis of all 5’D-II subunits contribute to the production of active 5’D-II in euthyroid cells, while only the latter process contributes to production of active 5’D-II in hypothyroid cells. Thus, our initial finding that steady-state production rates of enzyme activity are unaffected by T₄ (6) suggests that the rate-limiting synthetic step is the same in the presence and absence of T₄.

Our current model of the regulatory events that modulate 5’D-II activity is shown in Fig. 8. The synthetic pathway includes the production of 5’D-II polyepitope and a putative activating factor that leads to translocation of the enzyme to the plasma membrane and activation of catalytic activity. This activating factor may be a component of 5’D-II itself or simply an accessory/chaperon protein. As shown in Fig. 8A, T₄ initiates inactivation of 5’D-II, located on the cytoplasmic surface of the plasma membrane (7), by promoting the binding of the enzyme to the F-actin cytoskeleton (6, 7). The enzyme is then transported to the endosomes by an actin-mediated mechanism (7, 8), presumably involving a molecular motor protein (25, 26). From the endosomes, 5’D-II is shuttled back to the synthetic pathway, where it is recycled back to its active state on the plasma membrane. In hypothyroid cells or in cells treated with transcriptionally active T₄, the 5’D-II polyepitope(s) are internalized by an actin-independent pathway (Fig. 8B) (6, 7). The 5’D-II-containing vesicles are then sorted through the endosomes to the degradation pathway, ending up in the lysosomes (8).

The routing of vesicles through the endosomes to degradative, recycling, and synthetic pathways is regulated by targeting signals provided by vesicle-associated proteins located on the vesicle membrane. These vesicle proteins include regulatory coat proteins (17, 20), small Ras-like GTP-binding proteins (17), and proteins isolated primarily from synaptic vesicles, including the synapsins and the calcium-binding synaptotagmin (27–29). The binding of these proteins to, or removal from, vesicles results in the targeting of the vesicles to their ultimate destination. One potential mechanism by which T₄ may regulate vesicle sorting is by binding to one of these regulatory proteins and thus modulating the targeting signals for the p29-containing vesicle. A potential candidate protein to be regulated by T₄ is the synaptic vesicle protein synaptotagmin, which participates in vesicle recycling in the nerve terminal (27, 28). In addition to promoting vesicle recycling, synaptotagmin also influences the morphology of the actin cytoskeleton (29, 30), two cellular events regulated by T₄ in astrocytes. In summary, the regulation of 5’D-II activity in cultured astrocytes suggests that thyroid hormone regulates the basic mechanisms by which cells synthesize, degrade, and recycle proteins.

**REFERENCES**

1. Leonard, J. L., and Visser, T. J. (1986) in Thyroid Hormone Metabolism (Hennemann, G., ed) pp. 189–229, Marcel Dekker, Inc., New York
2. Visser, T. J., Leonard, J. L., Kaplan, M. M., and Larsen, P. R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5080–5084
3. Visser, T. J., Kaplan, M. M., Leonard, J. L., and Larsen, P. R. (1983) J. Clin. Invest. 71, 992–1002
4. Safran, M., and Leonard, J. L. (1991) J. Biol. Chem. 266, 3233–3238
5. Leonard, J. L., Silva, J. E., Kaplan, M. M., Mellen, S. A., Visser, T. J., and Larsen, P. R. (1984) Endocrinology 114, 988–1004
6. Leonard, J. L., Siegrist-Kaiser, C. A., and Zuckerman, C. J. (1990) J. Biol. Chem. 265, 940–946
7. Farwell, A. P., Lynch, R. M., Okulicz, W. C., Convi, A. M., and Leonard, J. L. (1990) J. Biol. Chem. 265, 18546–18553
8. Farwell, A. P., DiBenedetto, D. J., and Leonard, J. L. (1993) J. Biol. Chem. 268, 5055–5062
9. Leonard, J. L., Farwell, A. P., Yen, P. M., Chin, W. W., and Stula, M. (1994) Endocrinology 135, 548–555
10. Farwell, A. P., and Leonard, J. L. (1989) J. Biol. Chem. 264, 20561–20567
11. Leonard, J. L. (1988) Biochim. Biophys. Res. Commun. 151, 1164–1172
12. Helenius, A., Siegel-Kaiser, C. A., and Zuckerman, C. J. (1990) J. Biol. Chem. 265, 16363–16368
13. Pryer, N. K., Wuestehube, L. J., and Schekman, R. (1991) Annu. Rev. Biochem. 60, 487–488
14. Ciechanover, A., Schwartz, A. L., Dautry-Varsat, A., and Lodish, H. (1983) J. Biol. Chem. 258, 9681–9689
15. Rothman, J. E., and Orci, L. (1992) Nature 355, 409–415
16. Rudnich, A., Muller, I., Woll, D., and Hubbard, A. (1983) Trends Biochem. Sci. 8, 245–249
17. Mueller, S. C., and Hubbard, A. L. (1986) J. Cell Biol. 102, 932–942
18. Ciechanover, A., Schwartz, A. L., Dautry-Varsat, A., and Lodish, H. (1983) J. Biol. Chem. 258, 16363–16368
19. Pryer, N. K., Wuestehube, L. J., and Schekman, R. (1991) Annu. Rev. Biochem. 60, 487–488
20. McCarty, K. D., and de Vellis, J. (1980) J. Neurocytol. 9, 33–39
21. McCarty, K. D., and de Vellis, J. (1978) J. Neurocytol. 7, 15–26
22. Damke, H., Klumperman, J., von Figura, K., and Braulkes, T. (1991) Cell Biol. 116, 1071–1080
23. Lippincott-Schwartz, J., Yuan, L., Tipper, C., Amherdt, M., Orci, L., and Rasmussen, P. (1991) Cell 67, 601–616
24. Danke, H., Klumperman, J., van Nieuwenhoven, G., and Braulkes, T. (1991) J. Biol. Chem. 266, 24829–24833
25. Farwell, A. P., and Leonard, J. L. (1992) Endocrinology 131, 721–728
26. Safran, M., Farwell, A. P., Rokos, H., and Leonard, J. L. (1993) J. Biol. Chem. 268, 14224–14229
27. Wolenski, J., van Roonem, G., and Mooseker, M. S. (1993) J. Cell Biol. 126, 557–563
28. Wolenski, J. S., Cheney, R. E., Forscher, P., and Mooseker, M. S. (1993) J. Exp. Zool. 267, 33–39
29. Vally, R. B., and Sheptner, H. S. (1990) Annu. Rev. Biochem. 59, 909–932
30. Farwell, A. P., de Camilli, P., Niermann, H., and Jahn, R. (1993) Cell 75, 1–4
31. Sudhof, T. C., and Jahn, R. (1994) Nature 368, 665–677
32. Kelly, R. B. (1993) Nature 364, 487–488
33. Feany, M. B., and Buckley, K. M. (1993) Nature 364, 537–540
34. Safran, M., Farwell, A. P., and Leonard, J. L. (1995) J. Biol. Chem. 261, 16363–16368