The Actin Cytoskeleton Mediates the Hormonally Regulated Translocation of Type II Iodothyronine 5'-Deiodinase in Astrocytes*

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Thyroid hormone, specifically thyroxine, alters cytoskeletal organization in astrocytes by modulating actin polymerization and, in turn, regulates the turnover of the short-lived membrane protein, type II iodothyronine 5'-deiodinase. In the absence of thyroxine, ~35% of the total cellular actin is depolymerized, and >90% of the deiodinase is found in the plasma membrane and not associated with the cytoskeleton. Addition of thyroxine promotes actin polymerization and decreases the depolymerized actin to ~10% of the total actin pool, induces binding of the deiodinase to F-actin, and promotes rapid internalization of the enzyme. These data provide direct evidence that the actin cytoskeleton participates in the inactivation pathway of the deiodinase by translocating this short-lived plasma membrane protein to an internal membrane pool.

Interactions between the cytoskeleton and membrane proteins are essential for normal cell functions, such as the maintenance of cellular shape, endo- and exocytosis, and cell-to-cell and cell-to-substrate attachment (1–3). An organized meshwork of proteins consisting of filamentous actin (F-actin) and actin-binding proteins is contiguous with the cytoplasmic surface of plasma membranes and appears to be responsible for stabilizing and controlling the mobility and regionalization of transmembrane proteins. In only a few cases has the molecular organization of these membrane-cytoskeleton interactions been fully characterized (4–7). Although much is known about the biochemistry of actin polymerization, a thorough understanding of the molecular dynamics of membrane-cytoskeleton interactions with respect to cellular physiology has yet to be resolved.

The function of the cytoskeleton is modulated by interactions that affect the polymerization of actin and tubulin, the monomeric subunits of the microfilaments and microtubules, respectively (8). Recently, we showed that thyroid hormone, specifically thyroxine, regulates actin polymerization in astrocytes grown in culture (9). Under thyroxine-deficient culture conditions, cells had a depolymerized actin cytoskeleton, with no change in total cellular actin content. Thyroid hormone replacement resulted in the rapid repolymerization of F-actin and restoration of the actin cytoskeleton.

Recent studies (9–15) indicate that the thyroxine-dependent regulation of type II iodothyronine 5'-deiodinase (5'D-II), a plasma membrane-bound enzyme, is an excellent model for the examination of cytoskeleton-membrane protein interactions. 5'D-II generates >80% of the bioactive T3 in the cerebral cortex (10). Enzyme levels respond dynamically to alterations in extracellular thyroid hormone concentrations in vivo and in vitro (11–14). This action of thyroid hormone is independent of the nuclear thyroid hormone receptor, occurring in the absence of either protein synthesis or gene transcription (14). Thyroxine is the major effector of these actions and is >100-fold more potent than T3 in altering 5'D-II levels (14, 15). Disruption of the actin cytoskeleton with cytochalasins abolishes the thyroxine-mediated degradation of 5'D-II in astrocytes (14, 15), suggesting that the actin cytoskeleton participates in the inactivation pathway(s) of this short-lived enzyme.

In the present study, we characterize the cell membrane-cytoskeleton interactions that are required for the hormonal regulation of 5'D-II inactivation. Using the alkylation affinity label BrAcT4 to identify the substrate binding subunit of 5'D-II (15), we demonstrate that this enzyme binds to F-actin and is internalized in a thyroxine-stimulated, time-dependent fashion. These data provide direct evidence that the translocation of 5'D-II to an internal membrane pool by the actin cytoskeleton is fundamental to the hormonal regulation of the turnover of this short-lived plasma membrane protein.

EXPERIMENTAL PROCEDURES

Materials—T3, anti-T3, antisera (radioimmunoassay grade), Triton X-100, Fiske and Subbarow reducer solution, adenosine 5'-monophosphate, β-glycerophosphate, phenolphthalein-glucuronic acid solution, β-methylumbelliferyl-β-D-galactoside, D-glucose 6-phosphate, and ammonium molybdate were obtained from Sigma. 3,3',5'-T3 and T4 were obtained from Henning GmbH and rT4, from Calbiochem. Na[125I] (~17 Ci/mg, carrier-free) was purchased from Du Pont-New England Nuclear. Anti-actin antisera were obtained from Biomedical Technologies, Inc. (Stoughton, MA), affinity-purified goat anti-rabbit IgG-rhodamine conjugate from Jackson Laboratories (West Grove, PA), Vectastain ABC kit and Texas Red-streptavidin conjugate from Vector Laboratories (Burlingame, CA), affinity-purified goat anti-rabbit IgG gold conjugate and gold enhancement kit from Bio-Rad.
and nitrocellulose (0.2 μm pore size) from Hoefer Scientific Instruments (San Francisco, CA). SDS and lithium laurel sulfate were obtained from Gallard-Schlesinger Industries, Inc. (Carle Place, NY). Percoll from Pharmacia, and dithiothreitol from Calbiochem. Dulbecco's modified Eagle's medium, antibiotics, Hank's solution, and 0.25% trypsin were obtained from Gibco, and supplemented calf serum (heat-inactivated) was from HyClone, Inc. (Logan, UT). Cultured astrocytes were obtained from Nunc (Mash Biomedical Products, Rochester, NY). All other reagents used were of the highest purity commercially available.

Sprague-Dawley rats were obtained from Charles-River Labs (Kingston, NY) and were mated at the University of Massachusetts Medical School animal quarters.

RESULTS

Culture Conditions—Disassociated cerebrocortical cells were prepared from 1-day-old neonatal rats as described previously (15) and grown in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C in Dulbecco's modified Eagle's medium supplemented with 15 mM sodium chloride, 2 mM magnesium chloride, and 10 mM HEPES, pH 7.4, 10% (v/v) calf serum, 50 units/ml penicillin, 0.25% trypsin were obtained from Gibco, and supplemented calf serum (heat-inactivated) was from HyClone, Inc. (Logan, UT). Cultured astrocytes after density gradient centrifugation of glial cell homogenates on 16% Percoll gradients. 5'-Nucleotidase, a marker of the plasma membrane, demonstrates a polymodal distribution in a density range of 1.034-1.048 g/ml, with the "buoyant" fractions (fractions 1-13), with acid phosphatase more prominent in "dense" fractions (fractions 16-20). The nuclei located at the top of the gradient most likely represent the Triton-soluble supernatants. Aliquots from the homogenates and Triton-soluble supernatants were added to a PAGE sample buffer. Proteins were denatured in a boiling water bath for 5 min and resolved on 12.5% SDS-PAGE gels. Radiolabeled proteins were visualized by radiography, and bands of interest analyzed by scanning densitometry.

Affinity Labeling—Affinity labeling was carried out as described previously (15), with the following modifications. Cells were incubated with 10.4 mM BrAcTl, 260 (cpm/fmol) in 10 mM dithiothreitol, 50 mM HEPES, pH 7.4, for 20 min, after which the labeling media were removed, and the cells were washed once in Hank's solution. Cells were then treated with either 10 mM trypsin, T₅, or no hormone in 0.1% bovine serum albumin, harvested by scraping, suspended in ice cold 160 mM sodium chloride, 20 mM sodium phosphate buffer, pH 7.4 (PBS), and collected by centrifugation.

Subcellular Fractionation—Harvested cells were resuspended in 250 mM sucrose, 20 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, pH 7.0, and lysed by one freeze/thaw cycle. Homogenates (500-1000 mg) were centrifuged (9000 × g, min) through self-forming 16% Percoll gradients (19). Gradient fractions (0.5 ml) were collected, sonicated, and aliquots suspended in a PAGE sample buffer (1% (v/v) SDS, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromphenol blue, 50 mM Tris-HCl, pH 6.8). Proteins were denatured in a boiling water bath for 5 min and resolved by 12.5% SDS-PAGE according to the method of Laemmli (20). Radiolabeled proteins were visualized by radiography, and bands of interest analyzed by scanning densitometry.

Subcellular Marker Enzymes—Aliquots from fractions obtained above were assayed for subcellular marker enzymes. 5'-Nucleotidase (EC 3.1.3.5, plasma membrane), glyoxalase-6-phosphatase (EC 3.1.3.3, endoplasmic reticulum), and acid phosphatase (EC 3.1.3.2, lysosomes) were assayed by the liberation of inorganic phosphate from AMP, &Galactosidase (EC 3.2.1.23, Golgi) was assayed fluorimetrically by the hydrolysis of phenolphthalein-4-glucuronide. S'D-II were denatured in a boiling water bath for 5 min and resolved by 12.5% SDS-PAGE according to the method of Laemmli (20). Radioautographed proteins were >90% pure with 125I the only major contaminant.

Actin Analysis—Actin was detected by Western analysis (25) using affinity-purified polyclonal rabbit anti-actin antibody after proteins were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose (0.2 μm pore size). Immune complexes were visualized by gold-conjugated anti-rabbit IgG and enhanced with silver lactate (26). Quantitation of actin was determined by scanning densitometry.

Fluorescence—Fluorescence was analyzed using a Zeiss Axioskop microscope equipped with an Olympus OM4 camera (automatic f-stop and shutter speed settings) and Kodak Ektachrome ASA 400 film for image acquisition. Specific subcellular localization of fluorescently labeled proteins was assessed using confocal microscopy (Bio-Rad MRC 500). Settings were chosen for the acquisition of images with a depth of field of approximately 0.6 μm (29).

Total cellular fluorescence was quantitated by scanning densitometry. In brief, epifluorescent images were obtained at magnifications of x200 and 1000. Each image was then divided into 10 equal sections, with the integrated optical density of each section determined individually. The individual integrated optical densities were then averaged, and the results are expressed as the integrated optical density of the entire image, in arbitrary units.

Miscellaneous Methods—Protein was determined using the method of Bradford (30) with human immunoglobulin as the protein standard.

RESULTS

Density Gradient Fractionation of Cultured Astrocytes—Fig. 1A shows the distribution of subcellular organelles from rat astrocytes after density gradient centrifugation of glial cell homogenates on 16% Percoll gradients. 5'-Nucleotidase, a marker of the plasma membrane, demonstrates a polymodal distribution in a density range of 1.094-1.040 g/ml, with the major peak in fraction 9 (density 1.041 g/ml). A similar polymodal distribution of 5'-nucleotidase (31) and a bimodal distribution of 125I-labeled plasma membrane proteins (32) has been observed in human fibroblasts after fractionation on Percoll or colloidal silica gradients. The results of Arnold (23) report a density ~1.040 g/ml for the plasma membrane, coinciding with the major peak (fractions 9 and 10) of 5'-nucleotidase.

Two populations of lysosomes are present, which others (23, 31-33) have termed "buoyant" and "dense" lysosomes. β-Glucuronidase is more prominent in the "buoyant" fractions (fractions 8-13), with acid phosphatase more prominent in the "dense" fractions (fractions 16-20). The lysosomal enzymes located at the top of the gradient most likely represent lysosomal breakage during freeze/thaw homogenization. The "buoyant" lysosomes overlap with markers for the golgi (β-galactosidase) and the endoplasmic reticulum (glucose-6-phosphatase) over a density range of 1.038-1.042, a region suggested by others to also contain Golgi-endoplasmic reticulum-lysosomal-derived structures (23, 31-33). The nuclei pelleted at the bottom of the gradient (data not shown).
Enzyme activities were assayed in 0.5-ml fractions obtained. F-actin was depolymerized by low ionic strength, and actin was detected by Western analysis after SDS-PAGE as described under "Experimental Procedures." Western blots were analyzed by scanning densitometry and the cellular distribution of actin separated into cytosolic (fractions 1-3, density 1.035 g/ml) and cytoskeleton (fractions 8-10, density 1.041 g/ml) pools. Each point is reported as the percent of the total cellular actin in the respective assays. The affinity-labeling technique detects both the catalytically active 5'II-II as well as inactive enzyme containing an intact substrate binding site, whereas 5'II-II activity as determined via the iodide release method (10) measures only the intact, functional enzyme.

The data agree with the earlier subcellular localization studies done with hypothryoid rat cerebral cortex using sucrose density gradient and differential centrifugation (34). In contrast, others, using differential centrifugation alone (35), suggest that 5'II-II resides in the endoplasmic reticulum in bovine anterior pituitary. This may represent tissue-specific differences, different cellular fractionation and assay procedures used, or, most likely, the overlap of cell organelle marker enzymes after crude fractionation.

**Subcellular Distribution of Actin**—Actin showed a bimodal distribution on 16% Percoll density gradients. Under thyroid hormone-deficient culture conditions, ~35% of the cellular actin was present in the cytosolic pool (fractions 1-3, peak density 1.035 g/ml), with the majority of the remaining actin present in fractions 9 (peak density 1.041 g/ml, cytoskeleton pool), comigrating with the major peak of 5'-nucleotidase (Table I, Fig. 1B). Triton X-100 treatment of the cytosolic pool, followed by centrifugation in order to separate the G-actin (Triton-soluble actin) from the F-actin (Triton-insoluble actin) (23, 35), showed that the cytosolic pool contains only monomeric and short polymers of G actin (data not shown). The cytoskeleton pool, containing ~65% of the total actin under thyroid hormone-deficient conditions (Table I), likely represents the F-actin present in the cytoskeletal stress disruption of the plasma membrane during homogenization.

Three prominent BrAc[125I]T4-labeled protein bands are identified after affinity labeling of thyroid hormone-deficient, BtzcAMP-stimulated astrocytes, one of which (27 kDa) is the substrate-binding subunit of 5'D-II (15). Shown in Fig. 1B is the subcellular distribution of affinity-labeled 5'D-II (p27) after fractionation on 16% Percoll gradients. Approximately 45% of the total p27 was found in fractions 9 and 10, comigrating with the major peaks of 5'-nucleotidase and the catalytically active 5'D-II. The remaining p27 appears in the surrounding fractions, containing an overlap of golgi, lysosome, and Golgi-endoplasmic reticulum-lysosomal-derived organelles, and in denser fractions comigrating with the "dense" lysosomes. This dense population likely represents inactive enzyme being processed through lysosomal pathways. The differences between the distribution of the affinity-labeled 5'D-II and 5'D-II activity represents differences in the respective assays. The affinity-labeling technique detects both the catalytically active 5'D-II as well as inactive enzyme containing an intact substrate binding site, whereas 5'D-II activity as determined via the iodide release method (10) measures only the intact, functional enzyme.

**TABLE I**

<table>
<thead>
<tr>
<th>Effect of thyroid hormone on the subcellular distribution of actin</th>
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Thyroid hormone-deficient astrocytes were treated with 10 nM thyroxine, T₄, or no hormone for 20 min, homogenized, centrifuged through 16% Percoll gradients (final density range: 1.02-1.10 g/ml), and 0.5 ml fractions obtained. F-actin was depolymerized by low ionic strength, and actin was detected by Western analysis after SDS-PAGE as described under "Experimental Procedures." Western blots were analyzed by scanning densitometry and the cellular distribution of actin separated into cytosolic (fractions 1-3, density 1.035 g/ml) and cytoskeleton (fractions 8-10, density 1.041 g/ml) pools. Each point is reported as the percent of the total cellular actin in the gradient and is the mean of at least two experiments, with results differing by <10%.

<table>
<thead>
<tr>
<th>Total actin</th>
<th>Cytosol</th>
<th>Cytoskeleton</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>No hormone</td>
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<td>64.7</td>
</tr>
<tr>
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<td>85.0</td>
</tr>
<tr>
<td>10 nM T₄</td>
<td>34.2</td>
<td>65.7</td>
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</table>
fibers and the submembranous meshwork.

To determine if the comigration of the cytoskeleton actin pool and the plasma membrane is due to membrane-actin attachments, the cell membranes were treated with Triton X-100 to solubilize cell membranes. Triton solubilization had no effect on the subcellular distribution of the two pools of actin on 16% Percoll density gradients (data not shown), indicating that any membrane-actin attachments existing in the intact cell had no effect on the buoyant density of actin during fractionation.

Effect of Thyroxine on the Subcellular Distribution of Affinity-labeled 5'D-II and Actin—Thyroxine has been shown to rapidly decrease 5'D-II levels by increasing the inactivation rate of the enzyme in both intact animals and cultured astrocytes, an action that is blocked by disruption of the actin cytoskeleton (9, 11-14). Depicted in Fig. 2 is the effect of short-term thyroxine replacement on the subcellular distribution of p27 and actin. After 20 min treatment with 10 nM thyroxine, ~60% of the p27 is found in fractions 9 and 10 with a concurrent decrease in the amount of p27 in fractions 8 and 11. Along with this shift, a new peak is apparently unmasked at fraction 12, containing ~10% of the p27. Because of the overlap of organelles in this region, it cannot be determined if this represents a redistribution of p27 within the plasma membrane or the transfer of p27 to another pool.

Thyroxine also shifted the distribution of actin from the cytosol into the cytoskeleton pool (10.5% remaining cytosolic, Table I, Fig. 2), indicating polymerization of the G-actin monomers to F-actin fibers. There is also a new pool of actin seen in fraction 12, containing ~10% of the total actin and comigrating with the small p27 peak noted above, suggesting that, indeed, there may be transfer of both p27 and actin to another intracellular pool. T3 did not promote the redistribution of either p27 (data not shown) or actin (Table I) during the short time periods examined.

Thyroxine-induced Binding of Affinity-labeled 5'D-II to F-actin—Triton solubilization of cells, followed by centrifugation, readily separates the insoluble fraction, containing F-actin fibers and the rest of the filamentous cytoskeletal elements, from the soluble fraction, containing G-actin (36, 37). Using Triton X-100 to solubilize affinity-labeled astrocytes, we examined the effects of thyroxine on 5'D-II-cytoskeleton interactions. The radioautogram in Fig. 3A shows that ~90% of p27 is Triton-soluble in the absence of thyroxine. Treatment with thyroxine caused the rapid shift of p27 to the Triton-insoluble pellet containing the cytoskeletal elements. Because total p27 is unchanged by these treatments, any p27 lost from the Triton-soluble fraction must shift to the Triton-insoluble fraction. By 20 min, ~60% of labeled p27 shifted from the Triton-soluble supernatant to the Triton-insoluble pellets (Fig. 3B). T4 was much less effective, causing only 10-15% of p27 to shift from the Triton-soluble fraction. The time course of the thyroxine-stimulated loss of p27 from the Triton-insoluble fraction (and shift to the Triton-insoluble fraction) paralleled the time course of inactivation of 5'D-II activity by thyroxine (i.e., ~16 min versus ~14 min, respectively, Fig. 3B). These data indicate that the binding of 5'D-II to F-actin is a key step in the thyroxine-dependent inactivation of the deiodinase and suggest that the redistribution of p27 and actin seen into fraction 12 in Fig. 2 may be due to the formation of F-actin-enzyme complexes.

Two other prominent affinity-labeled proteins (55 kDa, p55; 18 kDa, p18), as well as some minor bands, appear to move to the Triton-insoluble pellet in a thyroxine-dependent fashion, albeit to a lesser extent than p27. Repeated experiments demonstrated that p27, p55, and p18 were the only affinity-labeled proteins that consistently shifted to the Triton-insoluble fraction after treatment with thyroxine. The identities of p55, p18, and other minor affinity-labeled proteins are uncertain at present, as is their role in this thyroxine-dependent translocation of 5'D-II.

Because the Triton-insoluble fraction of cellular homogenates contains actin, tubulin, and other cytoskeletal proteins, we examined whether p27 was binding to microtubules or to F-actin. Repeating the above experiment (treatment with 10 nM thyroxine for 20 min) in the presence of 0.01 mM colchicine, which specifically disrupts the microtubules, had no effect on the thyroxine-dependent shift of p27 to the Triton-insoluble pellet, with 42% of the p27 remaining soluble. On the other hand, 69% of the p27 remained in the Triton-soluble fraction after dihydrocytochalasin B (10 μM) treatment, which selectively disrupts actin polymerization (28). These data indicate that the microtubules do not play a significant role in this process, whereas actin polymerization is required for the thyroxine-stimulated p27 translocation.

Examination of the effects of other metabolic inhibitors revealed that low temperature (4°C) is the only condition, other than treatment with cytochalasins, that effectively inhibited the transfer of p27 to F-actin with 72.8% of the p27 remaining in the Triton-soluble fraction. Low temperature has been reported to stabilize G-actin and to decrease the rate of actin polymerization under polymerizing conditions (38). Indeed, 4°C also blocked the thyroxine-stimulated repolymerization of the actin cytoskeleton, with 35% of the total
actin remaining in the Triton-soluble fraction as opposed to <10% remaining at 37 °C. Low temperature also blocked the thyroxine-stimulated inactivation of 5′D-II activity (data not shown). Neither the lysosomotropic agents NH₄Cl (10 mM) nor chloroquine (200 nM) or the endocytosis inhibitors monodansylcadaverine (100 nM) nor methylamine (10 mM) affected the thyroxine-induced binding of p27 to the actin cytoskeleton, consistent with their inability to block thyroxine-stimulated inactivation of 5′D-II (14).

**Immunocytochemical Examination of the Thyroxine-stimulated Inactivation of 5′D-II**—Since 5′D-II is affinity-labeled with the hapten BrAcT₄, an anti-thyroxine antibody was used to identify the covalently modified enzyme. The resultant hapten-immune complexes were visualized with a fluorescently labeled, species-specific IgG. This approach allows direct examination of the cellular location of selected affinity-labeled proteins, and, thereby, the translocation resulting from a thyroid hormone-modulated event.

Shown in Fig. 4 is the ability of the polyclonal anti-thyroxine antibody to identify BrAcT₄-labeled proteins. Specific immunoprecipitation of affinity-labeled proteins was observed, with p27 the most abundant protein identified (46% of the total labeled proteins). The anti-thyroxine antibody immunoprecipitated 19.1 and 19.4% of the p27 in the initial homogenate at a 1:10 and 1:100 dilution, respectively.

Indirect immunofluorescence using the anti-thyroxine antibody to identify BrAcT₄-labeled proteins revealed three patterns of fluorescence in thyroid hormone-deficient astrocytes (Fig. 5): (a) specific, dense, coalesced fluorescence associated with the majority of the astrocytes, (b) specific punctate fluorescence located within the less intensely stained cells, and (c) nonspecific, punctate non-cell-associated fluorescence. The cell-associated fluorescence was reduced markedly in unpermeabilized cells (Fig. 5D), indicating an intracellular location for these proteins.

Quantitation of this cell-associated fluorescence, using wide-
field microscopy and scanning densitometry as described under “Experimental Procedures,” indicated that the density of the immunocytochemically detected proteins varied directly with levels of 5′-D′II. Stepwise increases in the thyroxine concentration in the growth medium resulted in parallel decreases in cellular fluorescence density and 5′-D′-II activity with an EC50 of 0.5 nM (Table II). Similarly, depolymerization of the actin cytoskeleton by dihydrocytochalasin B in thyroid hormone-replete cells caused parallel increases in cellular fluorescence density and 5′-D′-II activity, with peak values observed after 60 min. These data indicate that p27 (5′-D′-II) is the most abundant protein detected by this immunocytochemical technique.

With images of individual cells acquired using a laser-scanning confocal microscope, the effect of iodothyronines on the subcellular distribution of 5′-D′-II was then examined. In the absence of thyroxine, the immunofluorescently detected 5′-D′-II was limited to the plasma membrane, localizing the enzyme to the cytoplasmic surface of the plasma membrane (Fig. 6A). Examining the actin cytoskeleton in thyroid hormone-deficient astrocytes with FITC-phalloidin staining reveals that the quantity of F-actin is decreased markedly with an absence of stress fibers (Fig. 6D). After 20 min of exposure to 10 nM thyroxine, the majority of the 5′-D′-II was internalized (Fig. 6B), and the F-actin stress fibers were restored (Fig. 6E). Treatment for 20 min with 10 nM T3 was ineffective in either promoting internalization (Fig. 6C) or restoring the F-actin stress fibers (Fig. 6F).

**DISCUSSION**

We have characterized the cytoskeleton-membrane protein interactions necessary for the hormonally regulated inactivation of the integral membrane protein type II iodothyronine 5′-deiodinase (5′-D′-II) in astrocytes. Under hypothyroid culture conditions, ~90% of 5′-D′-II is solubilized by Triton X-100, demonstrating that this plasma membrane-bound enzyme is not associated with the actin cytoskeleton, which is lacking the F-actin stress fibers. Replacement with thyroxine rapidly mobilizes the cytosolic actin pool to restore the F-actin stress fibers and induces the binding of 5′-D′-II to F-actin at a rate equal to the thyroxine-dependent inactivation rate of 5′-D′-II. Once bound to the actin cytoskeleton, the 5′-D′-II-F-actin complex is then internalized rapidly. Thyroxine is the major effector of these actions, with T3 ~100-fold less effective. These data link two extranuclear actions of thyroid hormone, namely the thyroxine-dependent inactivation of 5′-D′-II (14) and the thyroxine-stimulated polymerization of F-actin (9), and provide direct evidence for the involvement of the actin cytoskeleton in the hormonally regulated turnover of an important membrane protein.

**FIG. 6.** The effects of thyroid hormone on immunofluorescently labeled 5′-D′-II and the actin cytoskeleton in BtEcAMP-stimulated astrocytes. Affinity-labeled astrocytes were fixed and permeabilized prior to immunofluorescent labeling. Each cell shown is representative of the entire cell population from one experiment, and each condition has been repeated in at least three separate experiments with similar results. A–C, confocal micrographs through the middle of representative cells immunofluorescently labeled after incubation with an anti-thyroxine antibody. D–F, indirect immunofluorescent micrographs of cells fluorescently labeled with FITC-phalloidin. A and D, thyroid hormone-deficient, BtEcAMP-stimulated cells. B and E, 20-min treatment of thyroid hormone-deficient cells with 10 nM thyroxine. C and F, 20-min treatment of thyroid hormone-deficient cells with 10 nM T3. Marker bar: 10 μm.

**TABLE II**

<table>
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<tr>
<th>Experiments</th>
<th>Total IP</th>
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<th>5′-D′-II activity units–mg protein–1</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 nM</td>
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<td>3024</td>
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</tr>
<tr>
<td>0.1 nM</td>
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</tr>
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<tr>
<td>0 min</td>
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<tr>
<td>90 min</td>
<td>1255</td>
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*Thyroid hormone-deficient, BtEcAMP-stimulated astrocytes were incubated for 24 h with increasing concentrations of thyroxine in 0.1% bovine serum albumin.

*10 μM dihydrocytochalasin B was added to euthyroid, BtEcAMP-stimulated astrocytes, and total immunofluorescence and 5′-D′-II activity was determined at the times indicated.
plasma membrane recycling in general, is less well understood. In one well characterized system, the internalization of the acetylcholine receptor appears to be more of a function of the microtubules rather than the microfilaments (4). In another, the translocation and reorganization of gastric parietal cell membrane upon stimulation by acid is mediated by the cytoskeletal proteins actin and spectrin, apparently independent of the microtubular network (44). In a third, the receptor-mediated internalization of thyrotropin-releasing hormone receptor complexes in GH3 cells appears to occur independently of both the microtubules and microfilaments (45).

The data presented in this paper suggest that one of the early events in the inactivation of 5'D-II is the binding of the enzyme to F-actin. The only agents that block the thyroxine-mediated 5'D-II inactivation (i.e. cytochalasins, low temperature) also prevent the thyroxine-stimulated repolymerization of the F-actin stress fibers and block the shift of affinity-labeled 5'D-II (p27) to F-actin. Once the enzyme is actin-bound, it is then translocated from the plasma membrane to internal pools. Classical lysosomal pathways do not appear to play an initial role in the thyroxine-dependent turnover of 5'D-II, as lysosomotropic agents and endocytosis inhibitors fail to block either enzyme inactivation (14) or the shift of p27 to F-actin in the presence of thyroxine.

Although studies in the rat GH3 pituitary tumor cell line (46) suggest that substrate-induced inactivation may play a role in the turnover of 5'D-II, it is clear from the present observations that the presence of thyroxine alone is insufficient to induce the rapid turnover of 5'D-II. A fully polymerized actin cytoskeleton, with intact F-actin stress fibers, appears to provide the machinery required for the translocation of 5'D-II to internal pools. It is possible that substrate-induced inactivation and actin-mediated translocation combine to accelerate the turnover of 5'D-II. In this model, thyroxine binding to the substrate-binding site of 5'D-II exposes an actin-binding site, thereby allowing the enzyme to bind to the cytoskeleton and be plucked from its membrane-bound site and internalized. However, more work remains to be done to identify the specific molecular mechanisms that initiate the binding of 5'D-II to F-actin.

How thyroid hormone regulates the plasma membrane-5'D-II-cytoskeletal interactions also remains to be determined. Since the function of the cytoskeleton is modulated by a variety of cytoskeletal-associated proteins, a likely mechanism of action would involve the interaction of thyroxine with one or more actin-binding proteins, followed by an increase in actin polymerization (8, 47). Several actin-binding proteins capable of influencing actin polymerization have been described in the brain (adducin, 48; ankyrin, 49; spectrin/fodrin, 50) and these may be targets for thyroid hormone binding, with subsequent association, or dissociation from the actin filaments. Further studies are necessary to characterize fully the specific events mediating thyroxine's stimulatory effect on actin polymerization.

In astrocytes, two other proteins bands accumulate the BrAcT4 affinity label in addition to the substrate binding subunit of 5'D-II (p27). These other proteins, specifically the 55- and 18-kDa polypeptides (p55 and p18, respectively), are possible candidates for the mediators of thyroxine's influence on actin polymerization, as thyroxine stimulates a shift of p18 and p55 to the Triton-insoluble, F-actin pellet. Further studies are necessary in order to evaluate the role of these proteins in the hormonal regulation of 5'D-II.

In summary, these studies demonstrate that the actin cytoskeleton plays a fundamental role in the turnover of short-lived membrane proteins, specifically 5'D-II, in astrocytes.

Further characterization of these membrane protein-cytoskeleton interactions will provide insight into the hormonal regulation of intracellular protein trafficking.

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The actin cytoskeleton mediates the hormonally regulated translocation of type II iodothyronine 5'-deiodinase in astrocytes.

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