Identification of a 27-kDa Protein with the Properties of Type II Iodothyronine 5'-Deiodinase in Dibutyryl Cyclic AMP-stimulated Glial Cells*

Alan P. Farwell§ and Jack L. Leonard

From the Departments of Nuclear Medicine, Medicine, and Physiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Type II iodothyronine 5'-deiodinase (5'D-II) catalyzes the intracellular conversion of thyroxine (T₄) to 3,3',5'-triiodothyronine (T₃), producing >90% of the bioactive thyroid hormone in the cerebral cortex. In cultured glial cells, expression of this enzyme is cAMP dependent. Exploiting the cAMP-dependent nature of this enzyme in these cells and utilizing N-bromoacetyl-3,3',5'-[125I]thyroxine (BrAc[125I]T₄) to affinity label cellular proteins, a 27-kDa protein with the properties of this enzyme was identified. Intact cells labeled with BrAc[125I]T₄ showed three prominent radio labeled bands of proteins of M₄, 55,000, 27,000, and 18,000 (p55, p27, p18, respectively) which incorporated ~80% of the affinity label. All three affinity-labeled proteins were membrane associated. One protein (p27) increased 5–6-fold after treating the cells for 16 h with dibutyryl cAMP; maximal specific incorporation of affinity label into the stimulated p27 was ~2 pmol/mg of cell protein in intact cells. Alterations in the steady-state levels of 5'D-II resulted in parallel changes in the quantity of p27. In cell sonicates, the rate of enzyme inactivation by BrAcT₄ equalled the rate of affinity label incorporation into stimulated p27, whereas p55 and p18 showed little or no specific dibutyryl cAMP-stimulated labeling. Enzyme substrates T₃ and 3,3',5'-triiodothyronine (rT₃) specifically blocked p27 labeling, whereas T₄ and the competitive 5'D-II inhibitor EMD 21388 (a synthetic flavonoid) were much less effective. Iopanoate, an inhibitor of all deiodinase isozymes, was ineffective in blocking p27 labeling. Inhibition kinetics revealed that iopanoate was a noncompetitive inhibitor of dibutyryl cAMP-stimulated glial cell 5'D-II, suggesting that it interacts at a site distant from the substrate-binding site. These data identify a cAMP-inducible membrane-associated protein (p27) that has many of the properties of 5'D-II.

Type II iodothyronine 5'-deiodinase (5'D-II) is an integral membrane protein that catalyzes the intracellular bioactivation of thyroid hormone in the brain, anterior pituitary, and brown adipose tissue (1–10). More than 90% of the T₄ bound to cell nuclei in the cerebral cortex is derived from the 5'-deiodination of T₄ catalyzed by this enzyme. Cerebrocortical levels of 5'D-II are increased 5–10-fold in hypothyroidism and decreased 80–90% in hyperthyroidism, and these changes constitute a homeostatic mechanism by which constant intracerebral T₃ levels are maintained. Although the regulation of this cerebrocortical enzyme has been studied intensively, isolation and purification have been hampered by the instability of the detergent-solubilized enzyme, the low levels of 5'D-II found in the brain, and the cellular heterogeneity of the central nervous system.

The distribution of 5'D-II among the major classes of brain cells has been examined in several laboratories. Leonard and Larsen (11) demonstrated that 5'D-II activity was found predominately in neurons prepared from primary dispersions of fetal brain cells. Subsequently, St. Germain (12) reported 5'D-II activity in the neuroblastoma cell line, NB4A3. Still others (13, 14) have proposed that gial cells are a source of 5'D-II activity, although prolonged serum deprivation was required to observe modest levels of catalytic activity. Recently, Leonard (15) and others (16) demonstrated that BrCAMP-stimulated glial cells contained abundant 5'D-II activity and that these stimulated cells exhibited all of the regulatory pathway(a) that modulate 5'D-II in vivo (15). This cell culture system provides a readily available source of cells for the identification and characterization of 5'D-II.

Affinity labeling of 5'D-II would eliminate the need for maintaining catalytically active enzyme during purification and characterization studies. Alkylating N-bromosacetyl derivatives of thyroid hormone have been used by others to label covalently several serum thyroid hormone-binding proteins (17, 18), the nuclear T₃ receptor (19), and cellular binding proteins in several cell lines including A431 (20, 21) and GH3 (22). In addition, Mol et al. (23) have demonstrated that

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§ To whom correspondence should be sent: Division of Endocrinology and Metabolism, Dept. of Medicine, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Tel: 508-856-6244.

1 The abbreviations used are: 5'D-II, type II iodothyronine 5'-deiodinase; T₄, L-thyroxine; T₃, 3,3',5'-triiodothyronine; T₂, 3,3',5'-triiodothyronine; T₃, L-thyroxine; BrAcT₄, N-bromosacetyl-L-thyroxine; BrAcT₄, N-bromosacetyl-3,3',5'-triiodothyronine; BrAc[125I]T₄, N-bromosacetyl-3,3',5'-triiodothyronine; [125I]triiodothyronine; EMD 21388, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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**BrAcT₃ irreversibly inactivates liver type I iodothyronine 5' deiodinase.**

Based on the biological properties of 5'D-II in glial cells, the following minimum criteria have to be fulfilled in order to identify the affinity-labeled enzyme. 1) Expression of the affinity-labeled protein(s) must be cAMP dependent. 2) The quantity of the cAMP-induced proteins(s) should change in parallel with T₄-dependent alterations in the steady-state levels of 5'D-II and increase after disruption of the actin cytoskeleton. 3) Affinity labeling of the protein(s) should be blocked by substrates and competitive enzyme inhibitors. 4) The protein(s) should be membrane bound. 5) The rate of enzyme inactivation by BrAcT₃ should equal the rate of label incorporation into one or more polypeptides.

In this study, we utilized BrAc[¹²⁵I]T₁ as an affinity label for the identification of 5'D-II. We have identified a cAMP-inducible membrane-associated 27-kDa protein (p27) that fulfills the criteria listed above. These data suggest that p27 is a component of the 5'D-II, presumably containing the substrate-binding site of the enzyme.

### EXPERIMENTAL PROCEDURES

**Materials**

T₃, T₂, BrAcT₃, hydrocortisone, glucose, and HEPES were obtained from Sigma; rT₄, and dihydrocholesterol-B were from Calbi-chem; and 3,3'-T₂ was from Henning GMBH. Na¹²⁵I (~17 Ci/mg, carrier free) was purchased from Du Pont-New England Nuclear. PTU was obtained from United States Biochemical Corp., IOP from Sterling Winthrop Research Institute, and EMS 21388 was generously provided by Dr. Josef Kohrle (Medizinishe Hochschule, Han- Anchor, Federal Republic of Germany). SDS and lithium lauryl sulfate were obtained from Gallard-Schlesinger Industries, Inc. Dulbecco's modified Eagle's medium, antibiotics, Hanks' solution, and 0.25% streptomycin. Cells were fed three times weekly and subcultured (2-4 X 10⁴ cells/cm²) when they reached confluence (7-10 days). Con- fluent cells from passages 2-4, containing >85% of the affinity-labeled enzymes, were used for the highest purity commercially available.

Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were mated at the University of Massachusetts Medical School animal quarters. BrAcT₃ and BrAcT₂, were synthesized according to published meth- ods (18). Briefly, 300 µl of a solution of the Na-hydroxycuscimide ester of bromoacetate in 1,2-dimethoxyethane (23.6 mg/ml) was added to 20 mg of L-T₃ or L-T₂, dissolved in a solution composed of 1 ml of 1,2-dimethoxyethane, 500 µl of dimethylformamide, and 80 µl of 14% triethanolamine. Incubations were done at 40 °C for 20-30 min, and the product was purified by preparative thin layer chromatography.

BrAc[¹²⁵I]T₁ and [¹²⁵I]rT₄ were prepared by iodination of BrAcT₃ or 3,3'-T₂, respectively, using the method of Weeke and Orskov (24). Radiolabeled iodothyronines were purified by chromatography on Sephadex LH-20 columns and their identities confirmed by high performance liquid chromatography analysis or, in a Synchropak C₁₈- RP column developed with a 0-90% acetonitrile gradient in 0.1% trifluoroacetic acid. The radiolabeled iodothyronines were >90% pure with ~2% the only major contaminant.

### Culture Conditions

Dissociated cerebrocortical cells were prepared from 1-day-old neonatal rats as described previously (15). Cells were seeded at a plating density of ~4 X 10⁴ cells/cm² in 75-cm² flasks 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 bicar- bonate, 33 mM glucose, 1 mM sodium pyruvate, and 15 mM supplemented HEPES, pH 7.4, 10% (v/v) calf serum, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were used three times weekly and subcultured (~3 X 10³ cells/cm²) when they reached confluence (7-10 days). Con- fluent cells from passages 2-4, containing >95% astrocytes, identified as large flat polygonal shaped cells containing the astrocyte-specific marker, glial fibrillary acidic protein (25, 26), were utilized for experiments.

Cultures selectively enriched in astrocytes or oligodendrocytes were prepared according to the method of McCarthy and de Vellis (26). Oligodendrocytes were identified as small round cells with multiple fine processes which lacked glial fibrillary acidic protein. Growth and propagation of the astrocyte cultures were as described above; en- riched oligodendrocytes were used without further subculture. Cultures were made thyroid hormone deficient by incubating for 24 h in serum-free medium. Cultures grown in 10% serum were considered "euthyroid."

**Affinity Labeling with BrAc[¹²⁵I]T₁.**

Unless otherwise noted, confluent cell monolayers were preincubated with serum-free medium for at least 8 h to remove serum iodothyronines followed by a 16-h stimulation period with 1 mM BrAc-cAMP and 100 µM hydrocortisone—conditions that induce abundant steady-state levels of 5'D-II (15).

Whole Cells—Just prior to labeling, the stimulation medium was removed, and the cell monolayers were washed three times with Hanks' solution. Cells were then incubated with 0.4 nM BrAc[¹²⁵I]T₁ (2600 cpm/mg) in Hanks' solution, pH 7.0, harvested by scraping, and suspended in ice-cold 150 mM sodium chloride, 20 mM sodium phosphate buffer, pH 7.4. Where indicated, cells were permeabilized by adding saponin to a final concentration of 0.25% (w/v) in the labeling medium and incubated as above. Labeled cells were collected by centrifugation, resuspended in 10 mM HEPES, 10 mM dithiothre- itol, 50 nM EDTA, pH 7.0, and 100 nM PTU. Aliquots of cell sonicates (20-50 µg of protein) in PAGE sample buffer (1% (w/ v) lithium lauryl sulfate, 1% (w/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue, 50 mM Tris-HCl, pH 6.8) were denatured in a boiling water bath for 10 min. Proteins were then resolved on 10 or 12.5% SDS-PAGE gels according to the method of Laemmli (27). Radiolabeled proteins were visualized by autoradiography, and bands of interest were either cut and counted or analyzed by scanning densitometry. Results were expressed as either fmol/mg of protein (cut bands) or the integrated optical density of bands after autoradiography.

**Sonicates**—Stimulated glial cells were collected as above, prior to the affinity-labeling step, and sonicated in 10 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA (pH 7.0). Cell sonicates (25 µg of protein) were then incubated at 37 °C with 0.5 µM BrAc[¹²⁵I]T₁ (6700 cpm/ mucol) in a total volume of 30 µl and the labeling terminated by adding 7.5 µl of a 5 X PAGE sample buffer and cooling on ice.

### RESULTS

**5'D-II Assay**

5'D-II activity was determined in cell sonicates as described previously (1). In brief, cell sonicates (25 or 50 µg of protein) were incubated in a total volume of 100 µl composed of 100 mM potassium phosphate (pH 7.0), 1 mM EDTA, 20 mM dithiothreitol, 2 nM [¹³¹I]T₄ (500 cpm/mg), and 1 mM PTU for 30-60 min at 37 °C (10-15% deiodination is typically observed after a 1-h incubation). Radioiodide was separated from the remaining substrate on Dowex 50WX-2X columns equilibrated in 10% (v/v) acetic acid, and the resultant elute was counted. Samples were run in triplicate and results ex- pressed as units/mg of protein, where 1 unit equals 1 fmol of T₄ released/h.

### Miscellaneous Methods

Protein was determined using the method of Bradford (28) with human immunoglobulin as the protein standard.

Incubation of BrAc-cAMP-stimulated thyroid hormone-deficient glial cell cultures, consisting of 95% astrocytes and 5% oligodendrocytes (25, 26), with BrAc[¹²⁵I]T₁ resulted in the appearance of three prominent radiolabeled bands of proteins: M, 55,000, 27,000, and 18,000 (p55, p27, p18, respectively) with ~30% of the total affinity label found in p27 and ~25% each in p55 and p18 (Fig. 1A). Minor labeling was occasionally seen at M, 50,000, 43,000-46,000, 31,000, and 22,000. Astrocytes contained >85% of the affinity-labeled proteins; en- riched oligodendrocytes showed a similar pattern of labeled proteins.

BrAc[¹²⁵I]T₁ labeling was time dependent, reaching a plate- au at 15-20 min in intact cells, which remained constant for up to 60 min with the three major labeled proteins follow-
Effects of Br2cAMP on the Pattern of BrAc[T4]-labeled Proteins in Intact Cells—Depicted in Fig. 1B is the effect of Br2cAMP on the pattern of affinity-labeled proteins. Br2cAMP stimulation markedly increased BrAc[125I]T4 incorporation into p27 compared with unstimulated controls, although minor increases in p55 and p18 (<50% increase) were also observed. In thyroid hormone-deficient cells, the quantity of p27 after Br2cAMP treatment increased ~5-fold over that observed in unstimulated control cells grown in medium containing 10% serum. Br2cAMP treatment of cells grown in serum-containing medium resulted in a 1.5-fold increase in the quantity of p27 over that observed in control unstimulated cells.

The effects of Br2cAMP on the levels of both affinity-labeled p27 and 5'D-II activity are shown in Table I. As seen previously (15), type II 5'-deiodinating activity was undetectable in unstimulated cultures grown in medium supplemented with 10% serum, whereas Br2cAMP induced abundant activity, averaging 3600 ± 1000 units/mg of protein (mean ± S.D.), in thyroid hormone-deficient cultures. Br2cAMP stimulated proportional increases in p27 and 5'D-II activity. Nonspecific labeling, defined as affinity label incorporation in the p27 region in the absence of detectable 5'D-II activity, was found to be ~20–25% of maximal labeling. Correction for this nonspecific label incorporation revealed that thyroid hormone deficiency resulted in a 6-fold increase in p27 and a 9.5-fold increase in 5'D-II.

Estimates of the quantity of p27 were done by titration with increasing amounts of BrAc[125I]T4, in intact Br2cAMP-stimulated glial cells without permeabilization. Maximal incorporation of the affinity label was ~2.6 pmol/mg of protein in Br2cAMP-treated thyroid hormone-deficient cells (Fig. 2). Nonspecific label incorporation was ~0.4 pmol/mg of protein in unstimulated cells devoid of 5'D-II activity, leading to a specific incorporation estimate of ~2.2 pmol/mg of protein.

Effects of Altered Steady-state 5'D-II Activity on p27 Labeling in Intact Cells—Previous studies have shown that steady-state levels of 5'D-II in brain (3, 5, 6) and in Br2cAMP-stimulated glial cells (15, 16) are inversely related to the thyroid hormone levels. Shown in Fig. 3 are the effects of increasing concentrations of T3 and T4 on steady-state levels of 5'D-II and the quantity of p27. Cells were grown in serum-free medium containing increasing amounts of T3 or T4 with 0.1% bovine serum albumin for 24 h prior to stimulation with 1 mM Br2cAMP and 100 nM hydrocortisone. Steady-state levels of 5'D-II decreased as the concentration of T3 increased, with an EC50 of ~0.4 nM. Similarly, the quantity of p27 also decreased in a T3-dependent fashion (EC50 ~ 0.5 nM). Incubation with up to 100 nM T3 failed to alter either the quantity

### Table I

| Condition   | p27 labeling | 5'D-II activity |
|-------------|--------------|-----------------|
|             | Total        | Specific        |
| Serum       | 1.2          | 0               |
| Serum + Stimulation | 1.8          | 0.6             |
| Serum-free  | 2.2          | 1.0             |
| Serum-free + Stimulation | 4.6  | 3.4             |

Confluent glial cells were incubated overnight in either 10% serum or serum-free medium ± stimulation with Br2cAMP and hydrocortisone. Affinity labeling was carried out in intact cells with 0.4 nM BrAc[125I]T4; 5'D-II activity was determined in cell sonicates. Data are reported as the mean of at least three experiments that differed by <15%. Specific p27 incorporation equals the total labeling minus nonspecific labeling.

To ensure access of the affinity label to all cellular compartments, cells were treated with saponin. Cells permeabilized during the affinity-labeling period showed a ~50% increase in p27 labeling which was maximal at 0.25% saponin (Fig. 1A) despite a loss of ~40% of the total cellular protein. There were little or no changes in p55 or p18 after saponin treatment.
Affinity Labeling of Type II Iodothyronine 5'-Deiodinase

Fig. 2. Effect of Br2cAMP on maximal BrAcT4 incorporation into p27. Intact confluent glial cell cultures were affinity labeled with 0.4 nM BrAcT4 in the presence of an increasing concentration of cold BrAcT4. Incorporation into p27 was determined after analysis on 12.5% SDS-PAGE gels, and the results are displayed on a double-reciprocal plot. , cells incubated for 24 h in serum-free medium and 16-h stimulation with Br2cAMP and hydrocortisone; O, cells maintained in medium containing 10% serum. Each point is the mean of at least two experiments that differed by <10%.

Fig. 3. Effect of steady-state concentrations of T4 and T3 on incorporation of BrAcT4 into p27 and 5'D-II activity. Confluent glial cell cultures were incubated for 24 h in serum-free medium with various concentrations of T4 (□—□, ○—○) or T3 (▲, ▼) in 0.1% bovine serum albumin prior to a 16-h stimulation with Br2cAMP and hydrocortisone. Affinity labeling was carried out in intact cells with 10.4 nM BrAcT4; 5'D-II activity was determined in cell sonicates. Results are expressed as percent of p27 incorporation (mean, ~11.2 fmol/mg of protein) or 5'D-II activity (mean, ~3600 units/mg of protein) as determined in the absence of any compound.

Table II
Effect of a variety of compounds on specific p27 labeling and 5'D-II activity in Br2cAMP-stimulated serum-free glial cells

| Compound | Concentration (μM) | Specific p27 labeling % | 5'D-II activity (units/mg) |
|----------|-------------------|-------------------------|---------------------------|
| T4       | 0.001 0.01 1.0 10.0 | 76 62 18 18 | 88 41 10 3 |
| T3       | 1.0 10.0 | 100 40 | 92 41 |
| rT3      | 0.001 0.01 1.0 10.0 | 85 71 42 16 | 55 60 60 30 |
| BrAcT4   | 0.01 0.1 1.0 10.0 | 75 42 23 0 | 75 42 23 0 |
| PTU      | 10.0 50.0 | 100 100 | 100 100 |
| EMD 21388| 0.01 0.1 1.0 10.0 | 100 64 63 61 | 100 100 100 61 |
| IOP      | 0.001 0.01 0.1 1.0 | 100 100 100 100 | 95 86 27 0 |

Labeling and 5'D-II Activity in Intact Cells—To establish the specificity of the BrAcT4 labeling, competition between the affinity label and selected iodothyronines for p55, p27, and p18 was evaluated. As shown in Table II, the addition of...
increasing concentrations of T₄, rT₃, and T₃ selectively blocked affinity labeling of stimulated p27. Consistent with the previous estimate of nonspecific labeling, ~20% of the affinity label incorporated into p27 was not blocked by up to a 100,000-fold excess of any iodothyronine (data not shown). T₄ was the best competitor of p27 labeling; T₃ was the worst. Similarly, 5′D-II activity showed parallel decreases with the addition of increasing concentrations of T₄, BrAcT₄, and T₃ to cell sonicates with T₃ > BrAcT₃ >> T₃. Affinity labeling of p55 and p18 was unaffected by up to a 100,000-fold M excess of any of the compounds tested (data not shown).

Effect of 5′-Deiodinase Inhibitors on p27 Labeling and 5′D-II Activity in Intact Cells—EMD 21388, a synthetic flavonoid that inhibits 5′D-I in liver microsomes and hepatocytes in suspension (30, 31), was found to be a modest inhibitor of p27 labeling, with maximal inhibition at 40–50% (Table II). Similarly, EMD 21388 is a relatively poor inhibitor of 5′D-II activity with an EC₅₀ ~ 5 μM. Reaction kinetics as determined from double-reciprocal plots showed the flavonoid to be a competitive inhibitor with a Kᵣ (5 μM) equal to the EC₅₀.

Neither PTU, a selective inhibitor of type 1 5′-deiodinase (10), nor IOP, an inhibitor of all deiodinase isozymes (10), had any effect on affinity labeling of p27 under the standard labeling conditions. To examine whether restricted entry into the cell contributed to the failure of these compounds to inhibit p27 affinity labeling, saponin-permeabilized cells were preincubated with 100 μM PTU or 10 μM IOP for 20 min. As shown in Fig. 5A, pretreatment of permeabilized cells with these compounds failed to block label incorporation into any of the major radiolabeled proteins.

In contrast to the lack of effect of IOP on p27 labeling, this compound was a potent inhibitor of 5′D-II activity (IC₅₀ ~ 50 nM) (Table II). This discordance between the IOP inhibition of 5′D-II activity and the failure of this compound to block BrAc[125I]T₄ labeling of p27 led us to reexamine the kinetics of enzyme inhibition by IOP. In contrast to earlier work (7, 9, 32), IOP was found to be a noncompetitive inhibitor of 5′D-II in Bt₂cAMP-stimulated glial cells (Fig. 5B), suggesting that it acts at a site distant from the substrate-binding site.

BrAcT₄. Inactivation of 5′D-II Activity in Glial Cell Sonicates—BrAcT₄ is a potent inhibitor of 5′D-II activity, with an EC₅₀ ~ 50 nM (Table II). To establish the relationship between BrAcT₄-dependent enzyme inactivation and affinity labeling of p27, the kinetics of inactivation of 5′D-II activity were determined. Under conditions in which enzyme disappearance followed pseudo-first order kinetics, the inactivation rate constant (k = 0.26 min⁻¹) was equivalent to the rate of specific BrAc[125I]T₄ accumulation by p27 (k = 0.30 min⁻¹) in cell sonicates (Fig. 6A). As seen in Fig. 6B, specific p27 labeling in sonicates was maximal at 3–4 min, consistent with maximal inactivation of 5′D-II by BrAcT₄. The rate of BrAc[125I]T₄ incorporation into p55 in unstimulated cells was similar to that seen in cells containing induced enzyme activity, indicating that little or no specific Bt₂cAMP-stimulated affinity labeling was present. p18 labeling in sonicates was essentially undetectable.

DISCUSSION

In this study, we utilized the alkylating affinity label BrAc[125I]T₄ to characterize a cAMP-inducible 27-kDa protein (p27) in cultured glial cells, which fulfills the criteria necessary for the identification of type II iodothyronine 5′-deiodinase. Steady-state levels of both p27 and 5′D-II are thyroid hormone dependent, decreasing as the T₄ concentration in the growth medium increases, and the quantity of p27 increases in a time-dependent manner after blocking the
that p27 is a component of 5'D-I1 and contains the substrate-binding site of this important cerebrocortical enzyme.

FIG. 6. BrAcT3 effects upon 5'D-II activity and incorporation into p27 in Bt2cAMP-stimulated serum-free glial cell sonicates. A, the inactivation of 5'D-II by BrAcT3 was plotted against the incorporation of BrAc[125]T3 into p27. 5'D-II activity was determined after incubation of glial cell sonicates with 500 nM BrAcT3 at 37° C for varying times. Glial cell sonicates were affinity labeled by incubation at 37° C with 500 nM BrAc[125]T3. Specific p27 represents the difference between labeling in stimulated versus unstimulated cells. B, time course of incorporation of BrAc[125]T3 into stimulated p27 and p55. Labeling units: integrated OD x 103.

BrAcT3 inactivation of 5'D-II activity. These data suggest that p27 is a component of 5'D-II and contains the substrate-binding site of this important cerebrocortical enzyme.

N-Bromoacetyl derivatives have been utilized to affinity label a variety of T4-binding proteins. In serum, BrAcT3 labeling has led to the characterization and amino acid sequence of the presumed T3-binding site of thyroxine-binding globulin (17) and human serum prealbumin (18). In studies using BrAc[125]T3, a membrane-associated protein of Mr 55,000-58,000 has been identified in the human epidermoid carcinoma A431 cell line (20), Swiss 3T3-F4 mouse fibroblasts (21), and rat GH3 pituitary tumor cell line (22). Of these cell lines, GH3 cells contain 5'D-II (7), but a 27-kDa protein was not described. This most likely represents suboptimal labeling conditions to identify 5'D-II. These include using the T3 derivative instead of T4, labeling at 15° C, and incubating for prolonged periods of time, thus decreasing the probability of selectively labeling a short lived protein such as 5'D-II

Studies using photoaffinity labeling with underivatized T4 have identified a T4-binding protein of Mr 27,000 in human plasma and in cultured hepatoma (Hep G2) cells which has been characterized and identified as apolipoprotein A-I (35-37). A comparison of apolipoprotein A-I and glial cell Bt2cAMP-stimulated p27 fails to reveal similar characteristics. Apolipoprotein A-I has only been described in the liver, intestine, and the kidney (38-40), and its synthesis appears to be restricted to these organs. Apolipoproteins are secretory proteins, and there is no evidence that glial cell p27 is either soluble or secreted. Finally, expression of apolipoprotein A-I has not been reported to be cAMP dependent. These observations make it unlikely that glial cell p27 is related to apolipoprotein A-I.

A protein of Mr 25,000-27,000 has been identified in liver microsomes using BrAcT3 (23) and in liver and kidney microsomes using BrAcT3 and BrAcT3. This protein is reported to exhibit the essential properties of the substrate-binding subunit of type I iodothyronine 5'-deiodinase. 5'D-I differs from 5'D-II in substrate specificity, inhibitor profiles, reaction kinetics, and response to thyroid hormone excess or deficiency (1-3, 10). Our data indicate that the Bt2cAMP-induced p27 in glial cells is related to the type II isozyme and not to 5'D-I. Unstimulated glial cell cultures contain primarily 5'D-I and no detectable 5'D-II (11, 13-15). Bt2cAMP treatment induces the appearance of 5'D-II, whereas levels of 5'D-I are unchanged and are of relatively low abundance as compared with the stimulated enzyme. Glial cell p27 is induced in parallel with the 5'D-II, and labeling is not blocked by 5'D-I inhibitor PTU, even when the cells are coincubated with this compound. The possibility that the p27 in unstimulated cells is related to 5'D-I is unlikely, as levels of p27 are present even in the presence of 1,000-fold excess of the substrate for the enzyme, conditions that should totally protect the 5'D-I substrate-binding site from affinity labeling. At present, both isozymes appear to have a component of Mr ~ 27,000, but the interrelationships between these two proteins are as yet undetermined.

The deiodinase inhibitor IOP has been described as exhibiting both competitive and noncompetitive inhibition kinetics upon 5'D-I and 5'D-II, depending upon which isozyme and which tissue is examined (7-9, 32). These differing results may indicate tissue-specific differences of the 5'-deiodinating enzymes. They also may represent differences in substrates utilized (T3 versus T4) and in the range of substrate concentrations evaluated. In Bt2cAMP-stimulated glial cells, IOP is an excellent inhibitor of 5'D-II activity. However, this compound failed to inhibit affinity label incorporation into p27, indicating a mechanism of inactivation of the enzyme different from the competitive inhibitors and substrates discussed above. Kinetic analysis confirmed this observation, as IOP is a noncompetitive inhibitor of glial cell 5'D-II. These data, combined with the lack of IOP inhibition of p27 labeling, indicate that IOP acts at a site distant from the substrate-binding site of 5'D-II in Bt2cAMP-stimulated glial cells.

We have demonstrated that steady-state levels of 5'D-II and p27 in Bt2cAMP-stimulated glial cells are dependent upon the T3 concentration of the growth medium. Previous reports have suggested that IOP also may act to decrease steady-state levels of 5'D-II in pituitary and GH3 cells (7). In our studies, exposure of glial cells to T3 under steady-state conditions resulted in a marked concentration-dependent decrease in both 5'D-II and labeled p27, indicating an actual decrease in the abundance of the enzyme. IOP, although decreasing 5'D-II activity, fails to alter the levels of p27, indicating no effect on levels of enzyme polypeptides. These data indicate that T3 and IOP act by different mechanisms to alter 5'D-II activity.

The two other major proteins labeled by BrAc[125]T3 in
glial cells (p18 and p55) are unrelated to 5’D-II, as incorporation of label under the conditions tested was entirely random. None of the published work with N-bromoacetyl derivatives of iodothyronines has identified a protein of M, 18,000, and its identity is as yet undetermined. As noted above, a major protein of M, 55,000-58,000 has been identified in several cell lines (20-22) using BrAc[125]I T4. Further work is being done in our laboratory to identify and characterize this protein in glial cells.

In summary, we have utilized the affinity label BrAc[125]I T4 to identify a protein (27 kDa, p27) in cultured glial cells. Labeled p27 will also ease both the development of antisera and the definition of the hydrodynamic properties of 5’D-II. These results provide us with a useful model to investigate intensively the regulation of T4 metabolism in the brain.

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* M. Safran, personal communication.