Catalytic Activity of Type II Iodothyronine 5′-Deiodinase Polyepitope Is Dependent upon a Cyclic AMP Activation Factor*

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Type II iodothyronine 5′-deiodinase is an ~200-kDa multimeric enzyme in the brain that catalyzes the deiodination of thyroxine (T₄) to its active metabolite, 3,5,3′-triiodothyronine. In astrocytes, cAMP stimulation is required to express catalytically active type II iodothyronine 5′-deiodinase. The affinity ligand N-bromoacyetyl-L-T₄ specifically labels the 29-kDa substrate-binding subunit (p29) of this enzyme in cAMP-stimulated astrocytes. To determine the requirements for cAMP-induced activation of this enzyme, we optimized N-bromoacyetyl-L-T₄ labeling of p29 in astrocytes lacking type II iodothyronine 5′-deiodinase activity and examined the effects of cAMP on the hydrodynamic properties and subcellular location of the enzyme. We show that the p29 subunit is expressed in unstimulated astrocytes and requires 10-fold higher concentrations of N-bromoacyetyl-L-T₄ to achieve incorporation levels equal to those of p29 in cAMP-stimulated cells. Gel filtration showed that p29 was part of a multimeric membrane-associated complex in both cAMP-stimulated and unstimulated astrocytes and that cAMP stimulation led to an increase of ~60 kDa in the mass of the holoenzyme. In unstimulated astrocytes, p29 resides in the perinuclear space. Cyclic AMP stimulation leads to the translocation of p29 to the plasma membrane coincident with the appearance of deiodinating activity. These data show that cAMP-dependent activation of type II iodothyronine 5′-deiodinase activity results from the synthesis of additional activating factor(s) that associates with inactive enzyme and leads to the translocation of enzyme polyepitope(s) from the perinuclear space to the plasma membrane.

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1The abbreviations used are: 5′D-II, type II iodothyronine 5′-deiodinase; T₄, thyroxine; T₃, 3,5,3′-triiodothyronine; rT₃, reverse T₃; 3,3′,5′-triiodothyronine; BrAcT₄, N-bromoacyetyl-L-thyroxine; PAGE, polyacrylamide gel electrophoresis.
Cell Culture—Astrocytes were obtained from 1-day-old neonatal rat cerebral hemispheres as described previously (10). Astrocytes were grown in a humidified atmosphere of 5% CO₂ and 95% air in Dulbecco’s modified Eagle’s medium containing 15 mM sodium bicarbonate, 33 mM glucose, 1 mM sodium pyruvate, 15 mM HEPES, pH 7.4, 10% (v/v) supplemented bovine calf serum, 50 units/ml penicillin, and 90 μg/ml streptomycin. Cells were passaged weekly and used between passages 1 and 3. For all experiments, unless otherwise noted, maximal 5’-D-II activity was induced in confluent monolayers by growth in serum-free medium for 24 h, followed by an additional 16 h with 1 mM dibutyl cAMP and 100 nM hydrocortisone (10).

Affinity Labeling of 5’-D-II—BrAc[125I]T₄ was prepared by radiodiode

nization of N-bromomethyl-t-T₄, as described previously (13). Cells were affinity-labeled at 37°C with 1.3 mM BrAc[125I]T₄ (specific activity of 2200 Ci/mmol) in buffered Hanks’ solution containing 50 mM HEPES, pH 7.4, unless otherwise indicated. After a 20-min incubation, cells were washed free of unincorporated affinity ligand, scraped from the dish, and collected by centrifugation. Cells were resuspended in 10 mM HEPES, pH 7.0, containing 10 mM dithiothreitol, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (lysis buffer) and sonicated. Either cell lysates were used directly for SDS-PAGE analysis, or crude microsomal fractions were obtained by centrifuging the lysates at 250,000 × g through a 0.8 mM sucrose cushion. Microsomes were resuspended by trituration in lysis buffer and used as described below.

Gel Filtration of BrAc[125I]T₄-labeled p29—Microsomal preparations from affinity-labeled astrocytes were solubilized in 5 mM taurodeoxycholate, and the detergent extracts were clarified by centrifugation for 30 min at 105,000 × g. The resultant supernatant was separated on a 90 × 1.5-cm Sephacryl S-300 column equilibrated in 50 mM NH₄Ac, pH 7.0, containing 1 mM dithiothreitol, 0.1 mM EDTA, and 5 mM taurodeoxycholate at a flow rate of 10 ml/h, and 1-ml fractions were collected. The detergent-soluble extracts from microsomes isolated from 3 × 10⁶ cells were used for each separation, and 100-μl aliquots of selected fractions were analyzed directly by SDS-PAGE. The distribution of p29 was quantified either by densitometry or by counting the radioactivity with a β-counter. The column was standardized using thyroglobulin, β-amylose, rabbit IgG, β-galactosidase, ovalbumin, and cytochrome c with dextran blue and 3H₂O used for the void volume and total volume, respectively.

Peptide Digests—Affinity-labeled 29-kDa proteins from cAMP-stimulated and unstimulated astrocytes were isolated from microsomal preparations by SDS-PAGE on 8–14% gradient gels as described previously (3). The 29-kDa proteins were cleaved directly in the gel slices with Staphylococcus aureus V8 protease or cyanogen bromide using a modification of the Cleveland method (see Refs. 3, 17, and 18). Digestion products were separated on a 15% SDS-polyacrylamide gel. Peptide fingerprints were compared to the manufacturer’s instructions. Control

Characterization of BrAcT₄-labeled 29-kDa Protein(s) in Astrocytes—One criterion that established p29 as the substrate-binding subunit of 5’-D-II was the observation that the quantity of affinity-labeled p29 was directly proportional to 5’-D-II catalytic activity (8). However, in unstimulated cells lacking 5’-D-II activity, another 29-kDa protein was also weakly labeled with BrAcT₄. To characterize this latter protein, we established conditions that optimized the BrAcT₄ labeling of this T₄-binding protein. The effect of increasing concentrations of BrAc[125I]T₄ on affinity labeling of astrocyte polypeptides is shown in Fig. 1. At low concentrations, BrAcT₄ incorporation into the 29-kDa protein in unstimulated cells was only 15% of that observed for p29 in cAMP-stimulated astrocytes as reported previously (8). At increasing concentrations of affinity ligand, this differential labeling pattern was progressively overcame, and little or no difference in affinity labeling of the 29-kDa protein(s) was observed at concentrations greater than ~2 nM BrAc[125I]T₄. These data identify an affinity-labeled 29-kDa protein in astrocytes lacking 5’-D-II activity and establish the labeling conditions necessary to allow comparisons between this protein and the p29 protein in cAMP-stimulated cells.

To establish the relationship(s) between these two 29-kDa polypeptides, we used limited proteolysis and peptide fingerprinting. Cyclic AMP-stimulated and untreated astrocytes were affinity-labeled with 10 nM BrAcT₄ for 20 min, conditions that effectively label both p29 and the 29-kDa protein in unstimulated astrocytes (see Fig. 1). The 29-kDa proteins were then isolated by SDS-PAGE, fragments were prepared by
cells, p29 was associated with a complex that was estms of holoenzyme size (3). In contrast, in unstimulated associated with a complex of
chromatogram of p29 in unstimulated and cAMP-stimulated "Experimental Procedures." Shown in Fig. 3 is a representative populations was consistently observed (Table I). Since cAMP-
smaller than that in cAMP-stimulated cells. This difference in complex, cAMP-induced protein-protein interactions could re-
result in the formation of a functional enzyme by causing p29 to
associate with the other, yet to be identified subunits in the
holoenzyme. Since p29 is part of an
200-kDa multimeric
\[200,000 \text{ kDa}, \beta\text{-galactosidase (} \beta\text{-gal; } 130,000 \text{ kDa), ovalbumin (oval; } 44,000 \text{ kDa), and cytochrome c }\]
(cytos-C; 12,000 kDa). AU, absorbance units.
induced 5′-D-II catalytic activity requires both transcription and translation (10), these data suggest that cAMP induces the synthesis of an essential activating factor that associates with the inactive 5′-D-II complex. There was little or no evidence of p29 monomers or dimers in the chromatograms from either the cAMP-stimulated or unstimulated astrocytes, indicating that most, if not all, of p29 is contained in the multimeric holoenzyme.

Characterization of Anti-p29 Antisera—To develop a 5′-D-II-specific immunological probe, anti-p29 antibodies were generated against partially purified p29 from cAMP-stimulated cells as described under "Experimental Procedures." Control studies showed that the anti-p29 antibody was not directed against Thr per se since anti-p29 IgG failed to immunoprecipitate Tg (Table II). This eliminated any potential problems of the cross-reactivity of this antibody with other BrAcT4-labeled proteins.

The specificity of the anti-p29 antibody is shown in Fig. 4A. As expected, in control immunoprecipitations, anti-T4 IgG rec-
ognized all of the BrAcT4-labeled proteins (second lane) (11, 12), while anti-p29 IgG preferentially immunoprecipitated the p29 polypeptide (third lane). We then determined if anti-p29 IgG recognized the holoenzyme in its native environment. 5′-D-II-containing endosomes were prepared from cAMP-stimulated astrocytes by density gradient centrifugation as detailed pre-
viably isolated vesicles containing p29 (third lane), while normal rabbit IgG controls failed to enrich vesicles containing any BrAcT4-labeled protein (second lane). Consistent with previous reports (11, 12), both the p55 and p18 affinity-labeled proteins were also associated with the immunopurified endosome since the anti-p29 antibody does not recognize either p55 or p18 from detergent-solubilized preparations (see Fig. 4A). These data
indicate that the anti-p29 antibody recognizes both detergent-
 soluble and membrane-bound forms of the p29 subunit of
 D-II.

Immunocytochemical Localization of the p29 Subunit of
 D-II in cAMP-stimulated and Unstimulated Astrocytes— In
 cAMP-stimulated astrocytes, catalytically active 5’-D-II is a
 plasma membrane enzyme, and T₄ regulates 5’-D-II activity by
 initiating the translocation of the enzyme from the plasma
 membrane to the perinuclear space, where it is catalytically
 inactive (11, 12). Since the p29 subunit is constitutively ex-
 pressed and part of a multimeric complex in both cAMP-stim-
 ulated and unstimulated astrocytes, we determined whether
 subcellular location played a role in the cAMP induction of
 catalytically active 5’-D-II.

A panel of representative photomicrographs of p29 immuno-
 reactivity is shown in Fig. 5. Preimmune serum controls
 showed no specific immunostaining in cAMP-stimulated, 
 BrAcT₄-labeled astrocytes. Anti-p29 IgG yielded intense stain-
 ing in cAMP-stimulated, BrAcT₄-labeled astrocytes, presum-
 ably over the cell membranes since p29 is an integral mem-
 brane protein (8, 12). Interestingly, in the nonaffinity-labeled,
 cAMP-stimulated astrocytes, the immunoreactivity of p29 was
 much less than that in BrAcT₄-labeled cells, suggesting that
 the epitope recognized by anti-p29 IgG requires affinity label-
 ing for maximal exposure.

### Table I

| Run No. | Column | V₁-V₀ | p29 (Kᵥ) |
|---------|--------|-------|----------|
|         |        | (ml)  |          |
| 1       | I      | 51.5  | 0.25     |
| 2       | I      | 0.23  |          |
| 3       | II     | 0.29  | 0.11     |
| 4       | II     |       | 0.22     |

### Table II

| IgG              | Immunoprecipitated (%) |
|------------------|------------------------|
| Anti-p29         | 0.9                    |
| Preimmune rabbit | 3.1                    |
| Anti-T₄          | 48.7                   |

**Fig. 4.** Autoradiograms of immunoprecipitated BrAc[¹²⁵I]T₄-
 labeled astrocyte proteins. A, taurodeoxycholate-solubilized affinity-
labeled astrocyte proteins (L) were immunoprecipitated using anti-T₄
IgG (T₄) and anti-p29 IgG (p29), and the purified proteins were sepa-
rated by SDS-PAGE. B, Percoll fractions containing the endosomal pool
(E) were obtained as described under "Experimental Procedures" and
then incubated with either normal rabbit IgG-linked (IgG) or anti-p29-
labeled (p29) magnetic beads. The isolated vesicle proteins were resolved
by SDS-PAGE.

**Fig. 5.** Identification of p29 in control and BrAcT₄-labeled
 cAMP-stimulated astrocytes. Dibutyryl cAMP-stimulated astrocytes
 were grown in the absence of thyroid hormone and affinity-labeled as
 indicated. Immunocytochemistry was done as described under "Exper-
 imental Procedures" using preimmune rabbit serum (NRS) and anti-
p29 antisera in nonaffinity-labeled cells (−BrAcT₄) and affinity-labeled
 cells (+BrAcT₄).

Since p29 constitutes ~50% of the affinity-labeled protein in
 astrocytes (8) and the BrAcT₄-labeled proteins are readily recog-
nized by anti-T₄ antibodies, we compared the distributions of
 anti-T₄ and anti-p29 immunoreactivity in astrocytes. Shown in
 Fig. 6 are representative confocal micrographs of the effects of T₄
 on the subcellular distribution of immunoreactive p29 in
 BrAcT₄-labeled, cAMP-stimulated astrocytes. As previously re-
 ported (11, 12), anti-T₄ IgG shows punctate staining along the
 cell periphery. Since the nucleus of an astrocyte is elliptical
 with a long axis of 8-10 µm and the astrocyte, while polyg-
noidal in shape, is ~30 µm in diameter, the nucleus occupies only
 25–40% of the cross-sectional area of any given cell, and the
 remaining intracellular space is filled with other organelles
 and cell sap. Thus, anti-T₄ IgG immunoreactivity is concen-
 trated over the plasma membrane in the hypothyroid cAMP-
stimulated astrocyte, and little, if any, staining is present in
the cell interior (Fig. 6A). After 20 min of T4 treatment (Fig. 6B), the BrAcT4-labeled protein(s) were lost from the cell membrane and had migrated to the perinuclear space, with occasional punctates observed over the cell nucleus. Since p29 is a membrane-associated protein (8, 11, 12) and, when internalized, is a component of the endosomal vesicle, this pattern of immunoreactivity is consistent with the translocation of affinity-labeled 5'D-II from the plasma membrane to the endosomal pool. Parallel astrocyte cultures stained with anti-p29 IgG exhibited identical patterns of immunostaining in hypothaloid cells (Fig. 6C) and showed the relocation of the immunoreactive protein to the perinuclear space in the T4-treated astrocytes (Fig. 6D). These data confirm that anti-p29 IgG recognizes the BrAcT4-labeled p29 subunit of the 5'D-II holoenzyme in intact cells.

Anti-p29 antiserum was then used to determine the subcellular localization of this 5'D-II subunit in unstimulated astrocytes grown in thyroid hormone-free medium (Fig. 7). In unstimulated astrocytes, p29 is found in the perinuclear space (P), and little, if any, specific immunoreactivity is associated with the plasma membrane (see arrows). In contrast, in cAMP-stimulated cells, the majority of immunoreactive p29 is found associated with the cell periphery, presumably the plasma membrane (see arrows), and little remains in the perinuclear space. These data suggest that cAMP stimulation results in the translocation of p29 from the perinuclear space to the plasma membrane, coincident with the appearance of catalytically active 5'D-II.

**DISCUSSION**

In this study, we show that p29, the substrate-binding subunit of 5'D-II, is constitutively expressed and resides, along with other 5'D-II components, in membrane vesicles located in the perinuclear space of unstimulated astrocytes. Cyclic nucleotides induce the appearance of catalytically active 5'D-II coincident with translocation of p29 to the plasma membrane. In addition, a cAMP-activating factor(s) is synthesized and becomes associated with the other enzyme components that are stored in vesicles located in the perinuclear space.

The identification of p29 as the substrate-binding subunit of 5'D-II was based upon multiple criteria, including a direct proportionality between the quantity of BrAcT4-labeled p29 and 5'D-II activity and the reciprocal relationship between inactivation and p29 labeling. In fact, the rate of enzyme inactivation is identical to the rate of affinity labeling of the p29 subunit (8). At the concentrations of BrAcT4 used in the early studies (8), minimal background labeling of 29-kDa protein(s) was observed in catalytically inactive cells. In this study, we show that cAMP increases the avidity of p29 for BrAcT4 by >10-fold. This increase in apparent binding affinity may be
due, in part, to the translocation of the enzyme to the cell surface in close proximity to the sites of entry of the affinity ligand and/or to the low $K_m$ for T₄ of the catalytically active enzyme (21).

The presence of the p29 subunit of 5′-D-II in astrocytes lacking the catalytically active enzyme was confirmed by peptide mapping, gel filtration, and immunocytochemistry. Peptide fingerprints of the p29 proteins in unstimulated and cAMP-stimulated astrocytes were identical. Gel filtration revealed that p29 was a component of a larger membrane-associated complex and was not present as a monomer or dimer in either the unstimulated or cAMP-stimulated astrocyte. These data show that the substrate-binding subunit of 5′-D-II is constitutively synthesized and integrated into vesicular storage membranes of the cell. Interestingly, cAMP induction of catalytically active 5′-D-II was accompanied by an increase in the molecular mass of this p29-containing complex, suggesting that an additional cAMP-activating factor(s) is responsible for the generation of active 5′-D-II.

The molecular events responsible for the cAMP-dependent generation of 5′-D-II activity in astrocytes are slowly emerging. Cyclic AMP induces expression of an activating factor that associates with the inactive 5′-D-II complex in storage vesicles and results in an increase in holoenzyme size. This is also accompanied by a marked difference in the subcellular location of the enzyme. Whether the cAMP-induced activation factor is integrated into the 5′-D-II complex prior to translocation to the plasma membrane or becomes associated with a 5′-D-II complex that is continually recycled to the plasma membrane is unknown. However, little, if any, immunoreactive p29 is located in the plasma membrane of unstimulated astrocytes, suggesting that recycling of the “inactive” 5′-D-II complex is minor. Thus, the cAMP-activating factor is required for the enzyme to relocate to its site of action at the plasma membrane.

The regulation of 5′-D-II activity in cultured astrocytes is a complex process that requires cAMP-induced protein synthesis, cAMP-stimulated translocation of an inactive 5′-D-II complex from intracellular storage pools to the plasma membrane, and microfilament-based endocytosis. In T₄-deficient astrocytes, the biological half-life of the catalytically active enzyme is identical to that of the p29 polypeptide (see accompanying paper (31)). However, in T₄-replete cells, p29 is rapidly internalized by endocytosis, and the biological half-life of catalytically active 5′-D-II is equal to the rate of p29 endocytosis, while the degradation of the p29 polypeptide remains unchanged under hormone-free or hormone-containing conditions (11). Since the cellular levels of p29 are not regulated by thyroid hormone and p29 is present in astrocytes that do not catalyze 5′-deiodination, then regulated expression of this substrate-binding subunit is not the mechanism by which the levels of catalytically active 5′-D-II are controlled. Moreover, constitutive expression of p29 indicates that the p29 subunit is not the essential cAMP-induced polypeptide. From previous studies, we know that the cAMP-stimulated appearance of 5′-D-II activity requires the synthesis of an essential protein(s) (10), and it appears that this cAMP-activating factor may be the short-lived protein essential for 5′-D-II catalysis. The demonstration that an essential cAMP-dependent factor(s) is required to activate the enzyme and target it to its site of action on the plasma membrane identifies one of the other 5′-D-II subunit polypeptides required for functional 5′-D-II. In vivo, both the pineal gland (22–24) and the limbic system (25) in the brain show catecholamine-induced increases in 5′-D-II activity, suggesting that cAMP is likely to play an important role in regulating the expression of a polypeptide that is essential for 5′-D-II catalysis, both in vivo and in cell culture.

Brain 5′-D-II is the largest of the deiodinases, with a calculated molecular mass of 200 kDa (3). Despite the recent cloning of an ~30-kDa frog selenoenzyme with kinetics similar to 5′-D-II (26), previous biological and biochemical evidence demonstrates that in the mammalian brain, 5′-D-II is not a selenoenzyme (27–30). Two of the required subunits of 5′-D-II are now known, the p29 substrate-binding subunit and an ~60-kDa cyclic AMP-inducible factor. Whether the essential cyclic AMP-inducible factor is an integral part of the enzyme or is merely required to target the enzyme to the plasma membrane remains to be established.

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