Cloning, Expression, and Functional Characterization of the Substrate Binding Subunit of Rat Type II Iodothyronine 5′-Deiodinase*

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Type II iodothyronine 5′-deiodinase catalyzes the bioactivation of thyroid hormone in the brain. In astrocytes, this ~200-kDa, membrane-bound enzyme is composed of at least one p29 subunit, an ~60-kDa, cAMP-induced activation protein, and one or more unidentified catalytic subunit(s). Recently, an artificial type II-like selenodeiodinase was engineered by fusing two independent cDNAs together; however, no native type II selenodeiodinase polypeptide is translated in the brain or brown adipose tissue of rats. These data suggest that the native type II 5′-deiodinase in rat brain is unrelated to this artificial selenoprotein. In this report, we describe the cloning of the 29-kDa subunit (p29) of type II 5′-deiodinase from a λzapII cDNA library prepared from cAMP-induced astrocytes. The 3.3-kilobase (kb) cDNA encodes an ~30-kDa, 277-amino acid long, hydrophobic protein lacking selenocysteine. Northern blot analysis showed that a 3.5-kb p29 mRNA was present in tissues showing type II 5′-deiodinase activity such as brain and cAMP-stimulated astrocytes. Domain-specific, anti-p29 antibodies specifically immunoprecipitated enzyme activity. Overexpression of exogenous p29 or a green fluorescence protein (GFP)-tagged p29 fusion protein led to a >100-fold increase in deiodinating activity in cAMP-stimulated astrocytes, and the increased activity was specifically immunoprecipitated by anti-GFP antibodies. Steady-state reaction kinetics of the enzyme in GFP-tagged p29-expressing astrocytes are identical to those of the native enzyme in brain. Direct injection of replication-deficient Ad5-p29GFP virus particles into the cerebral cortex of neonatal rats leads to a ~2-fold increase in brain type II 5′-deiodinating activity. These data show 1) that the 3.3-kb p29 cDNA encodes an essential subunit of rat type II iodothyronine 5′-deiodinase and 2) identify the first non-selenocysteine containing subunit of the deiodinase family of enzymes.

The enzyme-catalyzed deiodination of thyroxine (T4) in vivo generates the bioactive thyroid hormone, T3, and is the essential first step in the mechanism of thyroid hormone action. In the brain, type II iodothyronine 5′-deiodinase (D2) generates up to 75% of the T3 found within the cell and plays a key role in regulating intracellular T3 levels (1–8). Brain D2 is very short-lived in vivo and in cultured astrocytes with a t1/2 ranging from 10 to 20 min (9–11), and cellular levels of the enzyme are dynamically regulated by both T4 and 3,3′,5′-triiodothyronine (rT3) but not T3 (10, 12–15).

D2 belongs to a family of membrane-bound deiodinating enzymes. D1 (EC 3.8.1.4) and D3 are composed of 27- to 30-kDa selenoprotein subunits encoded by different mRNAs. Each mRNA contains a bifunctional, in-frame UGA codon that signals either selenocysteine (Sec) insertion or translation arrest (stop codon) depending on the cellular content of selenium. Unlike D1 and D3, the influence of selenium on D2 catalysis in vivo is unsettled; decreases in selenium intake that nearly eliminate D1 from the rat liver and kidney have only marginal effects on rat brain D2 activity (21, 26–28).

The discovery that frogs lack the D1 enzyme and express a deiodinase similar to D2 led to the cloning of a ~1.5-kilobase (kb) cDNA D2-like selenodeiodinase (SeD2) with an Sec codon and a 5′-UTR-located SECIS element (29). Injection of in vitro synthesized SeD2 mRNA in frog oocytes produced an ~30-kDa selenoprotein with D2-like catalytic properties and modest amino acid homology to the mammalian D1 (26).

Mammalian homologs of the frog SeD2 mRNA were cloned from rat brown adipose tissue (BAT) (30) and the human thyroid gland (31), but both cDNA clones did not produce functional enzymes due to the lack of the essential SECIS. However, appending a heterologous SECIS to the 3′-UTR of these inert clones generated artificial cDNA chimeras (SeD2SECIS) that yielded catalytically active ~30-kDa selenoproteins in transient expression studies (30, 31). These results were not unexpected, because the mammalian SeD2 mRNA(s) share significant homology with the catalytic core of D1 (30, 31) and adding a SECIS to the 3′-UTR of any mRNA facilitates Sec incorporation at in-frame UGA(s) (32, 33). Expressed sequence

5′-deiodinase; SeD2, selenocysteine type II iodothyronine 5′-deiodinase; p29GFP, green fluorescent protein-tagged p29; BrAcT4, N-bromosuccinimide-thyroxine; PAGE, polyacrylamide gel electrophoresis; p29, 29-kDa substrate binding subunit of type II iodothyronine 5′-deiodinase; TE- MED, N,N,N′,N′-tetramethyl ethylenediamine; βet-CAMP, dibutyryl cyclic AMP; BAT, brown adipose tissue; SecC, selenocysteine; SECIS, selenocysteine insertion sequence; UTR, untranslated region; kb, kilobase(s); m.o.i., multiplicity of infection.

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The abbreviations used are: T4, thyroxine; T3, 3,3′,5′-triiodothyronine; rT3, 3,3′,5′-triiodothyronine; D2, type II iodothyronine 5′-deiodinase; D1, type I iodothyronine 5′-deiodinase; D3, type III iodothyronine 5′-deiodinase; SeD2, selenocysteine type II iodothyronine 5′-deiodinase; p29GFP, green fluorescent protein-tagged p29; BrAcT4, N-bromosuccinimide-thyroxine; PAGE, polyacrylamide gel electrophoresis; p29, 29-kDa substrate binding subunit of type II iodothyronine 5′-deiodinase; TE-MED, N,N,N′,N′-tetramethyl ethylenediamine; βet-CAMP, dibutyryl cyclic AMP; BAT, brown adipose tissue; SecC, selenocysteine; SECIS, selenocysteine insertion sequence; UTR, untranslated region; kb, kilobase(s); m.o.i., multiplicity of infection.
tag (EST) data base scanning linked a potential SECIS element to the extreme 3'-end of the ~7.5-kb SeD2 mRNAs, but D2 synthesis from the reassembled human construct was poor (~7% of that obtained with an optimally located SECIS) (34), and the ~6-kb reassembled mice construct completely diverged (well upstream of the putative SECIS (35)) from the 5.3-kb SeD2 cDNA isolated from an astrocyte cDNA library (36).

Direct attempts to detect the native 30-kDa SeD2 polypeptide in rat tissues that express D2 failed, even though a functional SeD2 selenoprotein was easily identified in SeD2SECIS-transfected C6 astrocytoma cells (32). Instead of a full-length SeD2, the native 7.5-kb SeD2 transcript encodes a catalytically inert, 15-kDa polypeptide of unknown function (32), indicating that the UGA triplet in the native SeD2 mRNA signals translation arrest rather than SeC insertion. These findings suggest that D2 activity in rat brain is unrelated to SeD2 (32).

In this report we describe the cloning of the cDNA (GenBank accession number AF245040) encoding the p29 subunit of the D2 enzyme. The cloned 3.3-kb cDNA (p29) is found in neurons in vivo and encodes an essential iodothyronine binding subunit of D2 with a deduced molecular mass of 30.7 kDa.

**EXPERIMENTAL PROCEDURES**

**Materials—** All reagents were of the highest purity commercially available. Restriction endonucleases and DNA- and RNA-modifying enzymes were purchased from New England BioLabs (Beverly, MA). A femtomole DNA sequencing kit was obtained from Promega (Madison, WI). The AdpREC shuttle vector and the replication-deficient Ad5-βgal viral genome were gifts from T. Kowalik (University of Massachusetts Medical School); the replication-deficient Ad5-EGFP virus particles were a gift from X. Wang (Massachusetts General Hospital, Boston). [γ-32P]ATP (3000 Ci/mmol) and [α-32P]dTTP (800 Ci/mmol) were purchased from NEN Life Science Products. [3' or 5'-125I]rT3 (-2200 Ci/mmol) and [3' or 5'-131I]rT3 (-2200 Ci/mmol) were prepared by radiiodination of 3,3,5-triiodothyronine and 3,3,5,3-triiodothyronine, respectively, as described previously (10). Synthetic oligonucleotides were prepared in-house or purchased from Life Technologies (Grand Island, NY). The λ zapII cDNA library kit, picoBlue immunoscreening kit, and Duroine membrane kit, and Duralose membranes were purchased from Stratagene (La Jolla, CA). All iodothyronines were of the L-configuration and were purchased from Henning Berlin GmbH. Dulbecco’s modified Eagle’s medium, antibiotics, Hanks’ buffered salt solution, glucose, and trypsin were obtained from Life Technologies; supplemented bovine calf serum from HyClone Laboratories; dibutylryc cyclic AMP (bt,cAMP) and hydrocortisone from Sigma; acrylamide and N,N′-methylenebisacrylamide from E. S. Bio; membranes were isolated from the EntoPV from the AdpREC from CLONTECH, and/or by monitoring the number of p29GFP-positive HEK293 cells. Replication-deficient Ad5 virus particles were purified from HEK293 cell lysates by CsCl gradient centrifugation and stored at ~105 virus particles/ml at ~70 °C until use.

**Immunocytochemistry—** Cells were seeded onto poly-l-lysine (10 μg/ml)-coated glass coverslips (22 × 22 mm) and grown for 1–4 days. cAMP-stimulated astrocytes expressing D2 activity were treated with 10 μM colchicine for 30 min to depolymerize the microtubular network and relax the cell borders. Cells were then fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton (v/v) in phosphate-buffered saline. Where indicated, rat tissues were fixed with 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin, and ~6-μm sections were prepared. P29 expression was identified using affinity-purified, domain-specific, anti-p29 antibodies for 2 h at 4 °C (0.1–1 μg/ml). The specificity of antibody labeling was determined using peptide-blocked IgG prepared by preincubating the antibody with a 100-fold excess of peptide for 1 h at room temperature. Immune complexes were visualized with either Texas Red-conjugated goat rabbit-anti-IgG. DNA Sequencing—Double stranded DNA sequencing was done by the dideoxynucleotide method of Sanger (42) using cycle sequencing and iterative primers. All sequence information was confirmed by sequencing both strands.

**Production of Domain-specific Anti-p29 Antibodies—** Rabbit antibodies were raised against a 21-amino acid, synthetic peptide corresponding to the C terminus of the deduced amino acid sequence of p29 (NH2-YAQEMAFEEATPVDSLGGEK-I, see Fig. 1B). An N-terminal tyrosine residue was included to facilitate dianisobenzol coupling to keyhole limpet hemocyanin, and for radioiodination. This domain-specific antibody was used for all studies except the initial expression cloning of p29. Where indicated, domain-specific anti-p29 IgG was purified by affinity chromatography using a p29 peptide-Affigel affinity matrix. In brief, primary antiserum was adsorbed to the peptide-Affigel-Gel affinity matrix. After washing, the peptide-Affigel-Gel matrix was collected and eluted with ~1 mg/ml IgG until use.

**Immunoprecipitation of D2 Activity (Pull-down Assay)—** Immunoprecipitation of catalytically active D2 was done at 4 °C for 1 h in a total volume of 100 μl. Precipitation reactions containing from 50 to 100 units of detergent-solubilized enzyme, 100 μl HEPES buffer (pH 7.4), 100 mM NaCl, 10 mM octyl glycoside, 10 μl of immunobilized pR-Protein A beads (RepliGen, Cambridge, MA), and either preimmune rabbit antibody or anti-p29 antisera ≥ excess blocking peptide. Immune complexes were removed by centrifugation, and the D2 activity remaining in the clarified supernatant was determined as described previously (38). One unit of D2 activity equals the release of 1 fmol of iodide/h.

**Construction of p29 Expression Plasmids—** Replication-deficient adenovirus constructs (Ad5-p29 and Ad5-p29GFP) were created using the coding region of p29 cDNA alone and a green fluorescence protein (GFP)-tagged p29 chimera (p29GFP) formed by appending GFP to the C terminus of p29. In brief, the 825-bp Fsp-Hinfl fragment (containing the p29 coding sequence) was excised from pBSK-p29, blunted with Klenow, and cloned into the EcoRI site of the AdpREC from CLONTECH.

**Immunoprecipitation of D2 Activity (Pull-down Assay)—** Immunoprecipitation of catalytically active D2 was done at 4 °C for 1 h in a total volume of 100 μl. Precipitation reactions containing from 50 to 100 units of detergent-solubilized enzyme, 100 μl HEPES buffer (pH 7.4), 100 mM NaCl, 10 mM octyl glycoside, 10 μl of immobilized pR-Protein A beads (RepliGen, Cambridge, MA), and either preimmune rabbit antibody or anti-p29 antisera ≥ excess blocking peptide. Immune complexes were removed by centrifugation, and the D2 activity remaining in the clarified supernatant was determined as described previously (38). One unit of D2 activity equals the release of 1 fmol of iodide/h.
Cloning of the 29-kDa Substrate Binding Subunit of Brain D2 (p29)—Polyclonal anti-p29 antibodies (anti-p29) raised against the purified, affinity-labeled 29-kDa subunit of rat astrocyte D2 (37, 38) were used to screen approximately 10^6 clones from a cAMP-stimulated astrocyte azapII cDNA library. Eight positive plaques were identified, all from a single mRNA species, and all contained a cDNA insert of ~3 kb. Clone 11.24, with a 3.3-kb insert, was used in all subsequent work. The nucleotide and deduced amino acid sequence of the ~3.3-kb p29 cDNA is shown in Fig. 1A, and a limited restriction map is depicted in Fig. 1B. Two consensus Kozak translation initiation sequences (43, 44) are present; the first beginning at nucleotide 223 and the second at nucleotide 319, and yield open reading frames extending to nucleotide 1045, which encodes proteins of 277 and 245 residues, respectively, with molecular masses of ~30.5 and 27.2 kDa. Both deduced proteins have pIs of 4.5.

Cell-free translation under context-dependent translation initiation conditions (43, 44) was done to confirm the assignment of the initiator methionine. Shown in Fig. 2 are the p29 immunoreactive polypeptides programmed by cell-free translation of in vitro synthesized, full-length p29 mRNA. As expected, a doublet of ~30 and 27 kDa translation products was synthesized confirming the assignment of nucleotide 223 as the most 5'-authentic translation start site.

Tissue Distribution of the p29 mRNA and Cellular Localization of p29 Polypeptide—Northern blot analysis was done to determine the tissue distribution of the p29 mRNA. As shown in Fig. 3, the 3.5-kb p29 mRNA was expressed in rat tissues that contain D2 activity such as brain (B) and βt,cAMP-stimulated astrocytes (A), but not in tissues lacking this isozyme such as liver (L), kidney (K), and skeletal muscle (M). Consistent with earlier work that showed that p29 was present in unstimulated astrocytes lacking D2 activity (37), the p29 mRNA was also present in unstimulated astrocytes at levels similar to those found in the cAMP-stimulated cells.

The tissue distribution of native p29 polypeptide and its relationship to catalytically active D2 was then determined using a domain-specific, anti-p29 antibody raised against the deduced sequence of the C terminus of p29. As shown in Fig. 4, immunoblots of untreated astrocytes (A), βt,cAMP-stimulated astrocytes (A+), and cerebral cortex (CC) revealed a doublet of specific, immunoreactive p29 protein, indicating that both AUG codons initiate translation in cell culture and in vivo. To establish the relationship between the cloned p29 and native D2, domain-specific, anti-p29 antisera were used to deplete p29 from detergent-soluble D2 preparations and measured the antibody-dependent loss of p29 on D2 activity. Detergent-soluble D2 activity was prepared from cAMP-stimulated astrocytes and SeD2 cells, and from microsomal fractions isolated from the BAT and the cerebral cortex of hypothyroid rats and incubated with anti-p29 antisera (1:100 dilution) for 6100-fold molar excess of blocking peptide. The quantity of residual, non-absorbed D2 activity was then determined. As shown in Fig. 5, immune depletion of p29 resulted in the loss of 60–95% (p < 0.02 for glial and BAT, p < 0.002 for brain versus the peptide-blocked controls) of the D2 activity from all native enzyme preparations. No antibody-dependent loss of D2 activity was observed from the extracts prepared from the engineered SeD2 cell. In control experiments, >98% of the soluble D2 activity remained when excess blocking peptide was present, indicating that the anti-p29 antisera did not directly inhibit the catalytic reaction. These findings establish that the domain-specific, anti-p29 antisera specifically recognizes the native, catalytically active D2 and confirm that p29 is a subunit of the native D2.

With the specificity of the anti-p29 antibody documented by immunoblot and immunoprecipitation studies, the presence of p29 in D2-containing tissues was evaluated using affinity-purified, anti-p29 IgG. As expected, abundant, specific p29 immu-
Fig. 2. Cell-free translation products programmed by in vitro synthesized p29 mRNA. Approximately 1 μg of pBSK-p29 cDNA was linearized by digestion with XbaI and transcribed in vitro using T7 RNA polymerase according to the manufacturer's instructions (Stratagene). 1 μg of in vitro transcribed p29 mRNA was translated using 35S-labeled Met and the in vitro Express translation kit (Stratagene) modified according to Kozak (44). Newly synthesized proteins were immunoprecipitated with 1 μg of affinity-purified, anti-p29 IgG, and immune complexes were collected on rProtein A-Sepharose (RepliGen, Cambridge, MA) beads. Immune complexes were eluted by heating to 100 °C in 2× Laemml sample buffer containing 10 mM 2-mercaptoethanol. Eluates and the original translation reaction were resolved on 12.5% SDS-PAGE gels under reducing conditions and dried, and an exposure was made to Kodak X-Omat AR5 radiographic film for 24 h at −70 °C.

Fig. 3. Northern blot analysis of the p29 content in selected rat tissues. Total RNA was isolated from cAMP-stimulated astrocytes (A+), unstimulated astrocytes (A−), cerebral cortex (B), liver (L), kidney (K), and skeletal muscle (M) as described under “Experimental Procedures.” Northern blot analysis was performed by standard techniques using 1.2% formaldehyde/agarose gels and 10 μg of total RNA. Blots were washed to high stringency (30 mM NaCl, 3 mM sodium citrate, 1 mg/ml SDS) at 65 °C for 15 min. Hybridization signals were visualized using Kodak X-Omat AR5 radiographic film. The 32P-labeled p29 cDNA probe was prepared from the ~825-base pair Fsp1-Hinfl fragment (nucleotides 218–1043, see Fig. 1B) according to the method of Feinberg and Vogelstein (45). D2 activity was determined as detailed under “Experimental Procedures.” 18 S RNA visualized by ethidium bromide staining.

Fig. 4. Immunoblot of native p29 in untreated (A), cAMP-stimulated astrocytes (A+), and rat cerebral cortex (CC). 25-μg aliquots of cell lysate and cerebral cortex protein were separated on a 12.5% SDS-PAGE gel under reducing conditions, transferred by electroblotting to Immobilon-P (Millipore, Bedford MA), and probed with a 1:1500 dilution (final) of domain-specific anti-p29 antisera, followed by peroxidase-labeled goat, anti-rabbit IgG conjugated to horseradish peroxidase and chemiluminescence detection (Lumiglo, Kirkegaard & Perry, Gaithersburg, MD).

Fig. 5. Immunoprecipitation of catalytically active D2 using domain-specific anti-p29 IgG. Detergent-soluble, catalytically active D2 was prepared as described under “Experimental Procedures.” Clarified extracts were incubated, in triplicate, in a total volume of 100 μl with 0.5 μg of affinity-purified, domain-specific anti-p29 IgG, in the absence (○) or presence (□) of 10 μg of C terminus (p29) blocking peptide, and 10 μl of rProtein A-Sepharose beads. D2 activity was determined in the antibody-clarified extracts. Data are reported as the means ± S.E., n = 3. *, p < 0.02; §, p < 0.002 when compared with the +peptide control.

Consistent with earlier work done in dispersed brain cell cultures (45). Effects of Overexpression of Exogenous p29 on D2 Activity in Cell Culture and in Vivo—Because earlier work (37) showed that the generation of catalytically active D2 in astrocytes requires at least two components, p29 and one or more cAMP-induced proteins, we used two replication-deficient adenovirus constructs (Ads-p29 and Ads-p29GFP) to introduce exogenous p29 in rat astrocytes and examined the effects of p29 overexpression on D2 activity. As expected, in the absence of cAMP stimulation, overexpression of a p29GFP chimera yielded abundant GFP-positive cells (data not shown) but no D2 activity (Fig. 8A). However, after cyclic AMP stimulation, both the Ad5-p29- and the Ad5-p29GFP-infected astrocytes showed a dramatic increase in D2 activity over that in control, Ad5-GFP-infected cells (Fig. 8A). To determine if the elevated D2 activity observed in p29GFP-expressing astrocytes was due to synthesis of the GFP-tagged p29, anti-GFP antibodies were used to immunodeplete the fusion protein from detergent-soluble cell extracts, and the effects of the loss of the exogenous p29GFP on D2 activity were examined. Anti-GFP IgG immunoprecipitated 76 ± 12% of the p29GFP fusion protein, as judged by immunoblot analysis (data not shown), and reduced D2 activity by a parallel 75 ± 6% (Fig. 8B). On the contrary, the loss of GFP
alone from detergent extracts of control, Ad5-GFP-infected, cAMP-stimulated astrocytes had no effect on D2 activity. To ensure that the p29GFP fusion protein could be affinity-labeled with N-bromocetyl-L-thyroxine (BrAcT₄), control, Ad5-GFP-infected cells, and Ad5-p29GFP-infected cells were stimulated with cAMP and affinity-labeled with 0.2 nM BrAc[¹²⁵I]T₄ (4000 cpm/fmol) as described previously (38, 46). Detergent extracts containing ~2000 cpm of protein-bound BrAc[¹²⁵I]T₄ were then incubated with 1 μg of anti-GFP IgG, and immune complexes were then collected on protein A beads. As shown in Fig. 8C, the p29GFP fusion protein accounted for almost 40% of the protein-bound affinity label, whereas little, if any, affinity label was associated with GFP alone. Because 30–40% of protein-bound BrAcT₄ is associated with native p29 in cell lysates of cAMP-stimulated astrocytes (38, 46), these findings indicate that the D2 activity present in p29GFP-expressing astrocytes is due to the exogenous p29 fusion protein.

As shown in Fig. 9 the quantity of functional D2 in p29GFP-expressing cells was directly related to the quantity of fusion protein synthesized. Stepwise increases in the number of Ad5-p29GFP virus particles added to the astrocyte monolayer led to a progressive increase in D2 activity and a proportional increase to the number of GFP-positive cells that was directly related to the quantity of immunoreactive p29GFP expressed per cell. At multiplicity of infection (m.o.i.) > 6, more than 98% of the astrocytes expressed the p29GFP fusion protein, and D2 activity was >100-fold that in uninfected controls.

We then examined the catalytic properties of D2 activity in p29GFP-expressing astrocytes using steady-state reaction kinetics in the presence of 1 mM propylthiouracil with both rT₃ and T₄ as substrates (39, 47). D2 activity in cAMP-stimulated astrocytes expressing the exogenous p29GFP showed a converging set of lines (data not shown) consistent with the sequential, two-substrate reaction expected for this isozyme using rT₃ as the substrate (39, 47). Limiting Kᵢ values determined from secondary replots of the data yielded a Kᵢ for rT₃ of 6.7 nm, and Kᵢ for diethiothreitol of 18 μM, that are in close agreement with those of the native D2 (39, 47). The Vₘ for the D2 activity in p29GFP-expressing astrocytes was 59,000 units/mg of protein. T₄ was an excellent competitive inhibitor of rT₃ deiodination catalyzed by the D2 activity in p29GFP-expressing astrocytes with a Kᵦ of 3.1 nm, in close agreement with previous results (39, 47) and the Kᵦ for T₄ of 4.1 nm determined from secondary replots using T₄ as the substrate.

The very short, biological half-life of native D2 in brain is another unique characteristic of this enzyme (10, 12, 15). As detailed in Table I, the biological half-life of D2 in cycloheximide-blocked cells (10, 11) was 18 min in both cAMP-stimulated astrocytes expressing GFP alone (control cells) and in cAMP-stimulated, p29GFP-expressing cells, in close agreement with that determined previously for native D2 in cAMP-stimulated astrocytes (10, 11). Assuming steady-state expression of the p29GFP fusion protein, production rates of catalytically active D2 in the p29GFP-expressing cells and in the GFP-expressing astrocytes were 1200 and 8 units/min, respectively, indicating a >150-fold increase in D2 synthesis in the p29GFP-expressing astrocytes. Thus, two of the key distinguishing characteristics of the native D2 enzyme, (i) the sequential reaction kinetics with Kᵢ for iodothyronine in the nanomolar range, and (ii) a short biological half-life, are also properties of the D2 activity resulting from overexpression of the p29GFP fusion protein.

Effects of p29GFP Expression on D2 Activity in the Cerebral Cortex of 12-Day-Old Rats—Finally, the relationship between p29 and native D2 activity in vivo was examined by introducing the GFP-tagged p29 in one cerebral hemisphere of neonatal rats using the replication-deficient Ad5-p29GFP virus. Ad5-p29GFP or control Ad5-GFP (~10⁶ virus particles) was injected into the left cerebral hemisphere of 4-day-old neonatal rats, and D2 activity was determined in homogenates of both the injected (left) and uninjected (right) hemispheres on day 12. As shown in Fig. 10, expression of p29GFP (panel A) led to a 2-fold increase in native D2 activity in the left hemisphere (panel B). Neither p29GFP (panel A) and nor increase in D2 activity (panel B) was found in either the contralateral cerebral hemisphere or in the cerebral hemispheres expressing the control GFP protein. These data confirm that p29 is an essential subunit of the native D2 enzyme in rat brain in vivo.
protein-bound BrAc\textsuperscript{[125I]}T4 and normal rabbit IgG (1 ng) washed free of unincorporated affinity label, and the cells were collected by centrifugation. Immune precipitation was done with 2000 cpm of Triplicate flasks (25 cm\textsuperscript{2}) of confluent astrocytes were infected with replication-deficient Ad5 constructs as described above. After cAMP and the cells were grown for 24 h. As indicated in the solid bars, cells were stimulated with 1 mM bt\textsubscript{2}cAMP and 100 nM hydrocortisone for 16 h in complete growth medium. Cells were then harvested by scraping and collected by centrifugation, and D2 activity was determined, in triplicate, using \(\sim 25 \mu g\) of cell lysate per assay tube. Data are reported as the means of closely agreeing (\(\pm 10\%\)) triplicate flasks. B, Immunoprecipitation of D2 activity in cAMP-stimulated astrocytes expressing GFP or the p29\textsubscript{GFP} fusion protein. Two 75-cm\textsuperscript{2} flasks of confluent astrocytes were infected with 5 \(\times 10^5\) Ad5-GFP or Ad5-p29\textsubscript{GFP} virus particles and stimulated with 1 ms bt\textsubscript{c}AMP as detailed above. Detergent extracts of the cell pellets were prepared as described under “Experimental Procedures,” and the cells were collected by centrifugation. Immune precipitation was done with 2000 cpm of protein-bound BrAc\textsuperscript{[125I]}T4, and normal rabbit IgG (1 ng) or anti-GFP IgG (1 ng) as described above. After 60 min at 4 °C, the Immunobeads were washed a total of 5 times by resuspension in 10 volumes of 100 mM NaCl, 100 mM HEPES buffer (pH 7.4) containing 10 mM octyl glycoside, and the final pellets were counted in a well type \(\gamma\) counter. \(\square\), normal rabbit IgG (1 ng); \(\triangle\), anti-GFP IgG (1 ng). Data are reported as the means \(\pm\) S.E. (\(n = 3\)).

**DISCUSSION**

Intracellular thyroid hormone deiodination regulates the levels of bioactive T\(_3\) in the central nervous system. D2 serves as the source of T\(_3\) in the brain and differs from the D1 isozyme in substrate specificity and inhibitor profiles (47–50), physicochemical properties (37, 40, 51), trace mineral requirements (21, 27, 32, 52–54), and regulation (3, 8, 10, 14, 37, 55, 56). In this report, we identify and characterize an essential substrate binding subunit of this key enzyme and the first non-selenocysteine-containing subunit of the deiodinase family of enzymes.

D2 was first identified by affinity labeling with BrAcT\(_4\), an alkylating analog of the D2 substrate (46). This substrate analog selectively labeled a 29-kDa protein (p29) with all of the properties of the substrate binding subunit of D2 (46). Both T\(_4\) and T\(_3\) specifically block the selective labeling of p29 and the affinity label-dependent loss of D2 activity, whereas the product, T\(_3\), had no effect on either affinity label incorporation or BrAcT\(_4\)-dependent inhibition of D2 activity (46). Rate inactivation studies confirmed that accumulation of BrAcT\(_4\) by p29 led directly to D2 inactivation (46), indicating that p29 was an essential subunit of the enzyme (14, 38, 57).

Expression cloning yielded the p29\textsubscript{GFP} DNA from an astrocyte cDNA library. This 29-kDa protein is encoded by a 3.5-kb mRNA that is found in all native tissues expressing D2 activity. Domain-specific, anti-p29 antibodies raised against synthetic peptides based on the deduced amino acid sequence of the C terminus of the p29 cDNA, selectively immune-depleted native D2 activity from detergent extracts of both the brain and BAT of rats. Importantly, cAMP stimulation was required be-
fore either exogenous p29 or the GFP-tagged p29 increased D2 activity in astrocytes. This finding is consistent with prior work showing that a 60-kDa cAMP-induced subunit required to assemble a catalytically active enzyme and to direct D2 to the plasma membrane (37). Together with the well characterized, T3-dependent intracellular trafficking of the native p29 polypeptide, the p29 GFP fusion protein provides a readily visualized reporter that can monitor intracellular trafficking of D2 in real time and allow the molecular events that regulate D2 levels in vivo to be characterized. Preliminary results indicate that the p29 GFP fusion protein shows thyroid hormone-dependent endocytosis with properties identical to those described for the D2 activity and native p29. Thus, the cloning of the p29 subunit of native D2 provides a key enzyme polypeptide for the characterization of the non-genomic events mediating D2 regulation and the first non-selenocysteine member of the deiodinase family of enzymes.

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