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Molecular Biological and Biochemical Characterization of the Human Type 2 Selenodeiodinase*

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ABSTRACT

Type 2 deiodinase (D2) is a low K_m iodothyronine deiodinase that catalyzes the removal of a single iodine from the phenolic ring of T_4 or rT_3. We sequenced and subcloned the open reading frame from a partial complementary DNA (cDNA) clone (2.1 kilobases) prepared by Genethon (Z44085) from a human infant brain cDNA library. The open reading frame encodes a putative 273-amino acid protein of 31 kDa with greater than 70% similarity to the *Rana catesbeiana* D2 protein. Transient expression of the cDNA produces a low K_m (5 nM for T_4; 8 nM for rT_3) propylthiouracil- and gold thioglucose-resistant 5′-deiodinase in 293-HEK cells. Human D2, like human type 1 (D1) and type 3 (D3) deiodinases, is a selenoenzyme, as evidenced by 1) the presence of two in-frame UGA codons (positions 133 and 266), 2) the synthesis of a 31-kDa ^{75}Se-labeled protein in D2 cDNA-transfected cells, and 3) the requirement for a 3′-selenocysteine incorporation sequence element for its translation. Unlike D1 and D3, we were not able to covalently label overexpressed D2 with *N*-bromoacetyl [^{125}I]T_3 or -T_4. We found that the human D2 messenger RNA is 7–8 kilobases and is expressed in brain, placenta, and, surprisingly, cardiac and skeletal muscle. Type 2 deiodinase activity was also present in human skeletal muscle. These results indicate that there are unique features of D2 that distinguish it from the two other selenodeiodinases. The expression of D2 in muscle suggests that it could play a role in peripheral, as well as intracellular, T_3 production. (*Endocrinology* **137:** 3308–3315, 1996)

THE FIRST STEP in thyroid hormone action is the removal of a single iodine from the phenolic ring of T_4 to form T_3. Two enzymes, the type 1 and 2 deiodinases (D1 and D2), catalyze T_4 activation. The D1 enzymes from several species have been cloned (1–4). All contain selenocysteine in the active site, and rat D1 is expressed in liver, kidney, thyroid, and pituitary (1). Iodothyronine deiodination by D1 is characterized by a K_m for T_4 of 1–2 μM and high sensitivity to inhibition by 6*n*-propylthiouracil (PTU; K_i, 0.2 μM) and gold thioglucose (GTG; K_i, ~7 nM) (5–7). The presence of a second outer ring deiodinase (D2) was first inferred from *in vivo* experiments showing that PTU does not block the inhibition of pituitary TSH release or the generation of pituitary nuclear T_3 after T_4 administration to hypothyroid rats (8). Later studies demonstrated that monodeiodination of T_4 by D2 has a K_m for T_4 of ~2 nM (much lower than that for D1) and is relatively insensitive to PTU (K_i, >1 mM) and GTG (K_i, ~2 μM) (7, 9, 10). D2 activity has been found in rat pituitary, the central nervous system, brown adipose tissue (BAT), human keratinocytes, and human placenta (11–13). The local intracellular conversion of T_4 to T_3 by D2 provides about 50% of the thyroid receptor-bound T_3 in rat pituitary (14) and BAT (15) and more than 75% of the nuclear T_3 in brain (16).

Recently, using a PCR-based technique, Davey *et al.* (17) isolated a complementary DNA (cDNA) encoding for a D2 enzyme from *Rana catesbeiana* tissues. Significant similarities of a portion of the 5′-untranslated region of this sequence were noted with a partial sequence from a 2.1-kilobase (kb) cDNA clone prepared from a human infant cDNA library by Genethon (Evry, France) and entered in GenBank (Z44085). We sequenced this clone and identified an open reading frame with close similarity to that of the *Rana* D2. Transient expression of the putative coding region of this cDNA in embryonic kidney cells (HEK-293) produced a protein with the enzymatic characteristics of D2. Human D2 (hD2) contains two in-frame UGA codons, and the transiently expressed enzyme can be labeled cotranslationally with ^{75}Se in culture. We found the 7.5-kb D2 messenger RNA (mRNA) in human brain and placenta, but not in liver, kidney, or pancreas. Surprisingly, we also found D2 mRNA in human cardiac and skeletal muscle, and we identified D2 enzyme activity in a sample of human skeletal muscle. There are areas of high similarity among the hD1, hD2, and hD3 enzymes, particularly in the amino acids surrounding the active site, indicating that these three deiodinases belong to a group of closely related selenoenzymes.

Materials and Methods

Partial D2 cDNA clone

We received the Z44085 cDNA (2.1 kb) derived from the infant brain cDNA from Genethon through the courtesy of Dr. Donald St. Germain, Dartmouth Medical School. This DNA contains the Z44085 sequence that was found be 74.5% identical (in 173 bp) to the *Rana* D2 cDNA. It was cloned into the *Hin*dIII-*Not*I sites of the Lafmid BA vector (18), and we used the same restriction enzymes to excise the entire 2.1-kb DNA from the vector and subclone it into the *Hin*dIII-*Not*I site of CDM-8 (19).

Northern blotting

A multiple tissue Northern blot (Clontech Laboratories, Palo Alto, CA) containing 2 μg polyadenylated [poly(A)$^+$] RNA from a number of

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normal human tissues was hybridized with a *Dra*I hD2 fragment containing the open reading frame. The filter was prehybridized for 3 h at 42 C in a solution containing 50% formamide [25 mM potassium phosphate buffer (pH 7.4), 5 × SSC, 5 × Denhardt's solution, and 50 μg/ml salmon sperm DNA]. Hybridization was performed in the same solution for 12–20 h in the presence of [^{32}P]CTP-labeled cDNA probe. We have previously probed this same blot with the hD3 and β-actin cDNAs (20).

Preparation of active D2 vectors for eukaryotic expression

Based on the sequence derived from the hD2 Lafmid BA vector, we prepared two oligos, sense (oligo 1, GCCACCATGGGCATCCTCAGCGTA) and antisense (oligo 2, TCTCTTATAATCATACCTTTAACC). Oligo 1 contains the optimal Kozak consensus sequence (GCCACC) immediately before the initiator ATG. We used these two oligos in a PCR reaction using the Z44085 Lafmid BA vector as template to obtain a 846-nucleotide PCR product corresponding to the entire hD2 coding region. Because a selenocysteine incorporation sequence (SECIS) element is required for incorporation of selenocysteine into selenoproteins (21), and our initial studies showed that the Z44085 cDNA clone did not contain this element (see *Results*), we prepared chimeric constructs in which D2 was inserted 5′ to the SECIS elements from other genes. In one construct, we inserted this DNA fragment upstream of the active SECIS element of the SelP selenoprotein. (*Xba*I-*Not*I fragment) (22). We subsequently subcloned both the wild-type and the PCR-generated D2-SelP chimeras into the CDM-8 vector (19) and also into the more potent expression vector D10 (23), 5′ to the SECIS element from either the hD3 or SelP genes (20, 22).

DNA transfections

To produce D2 protein for kinetic analyses, we expressed it transiently by introducing expression vectors containing the D2 cDNA into human embryonic kidney cells (HEK-293) in culture. Transfection of HEK-293 cells was performed by calcium phosphate precipitation, as described previously (24). These cells do not express D1, D2, or D3 activity after transfection with vector alone. Two days after transfection, cells were harvested, washed, and sonicated briefly in 0.1 M potassium phosphate-1 mM EDTA, pH 6.9 (PE buffer), containing 25 mM dithiothreitol (DTT). Internal transfection efficiency was monitored using thymidine kinase-GH as previously described (3).

Outer ring deiodinase assays

Cell homogenates were assayed for deiodination of [^{125}I]T_4 and [^{125}I]rT_3 (DuPont-New England Nuclear, Boston, MA) under varying conditions, as described for each experiment. Deiodinase assays contained 30–150 μg cell sonicate protein, varying concentrations of unlabeled T_4 or rT_3, 20 mM DTT, and 1 mM PTU in PE buffer in a final volume of 300 μl. Under standard assay conditions, reactions contained either 2 nM T_4 and 1 mM PTU or 0.1 nM rT_3, 1 mM PTU, and 20 mM DTT. Incubations were performed for 60 or 120 min at 37 C. $^{125}I^-$ was separated by trichloroacetic acid precipitation after the addition of horse serum as previously described (25). Deiodinations were linear with both time and protein under the conditions of the assay, and quantities of protein were adjusted to consume less than 30% of the substrate. Studies of human skeletal muscle were performed under a protocol approved by the internal review board of the Brigham and Women's Hospital.

Incubation with N-bromoacetyl [^{125}I]T_3 or T_4

N-Bromoacetyl-[^{125}I]T_3 and -T_4 (BrAcT_3 and BrAcT_4) were synthesized from [^{125}I]T_3 and [^{125}I]T_4 (DuPont-New England Nuclear; SA, 1200 μCi/mg) and bromoacetyl chloride according to previously described methods (26). The product was purified on Sephadex LH-20 by elution with ethanol. Affinity labeling was performed by incubating 60–150 μg sonicate or microsomal protein in 50 μl PE-10 mM DTT for 10 min at room temperature, followed by exposure to 0.1 μCi BrAcT_3 or BrAcT_4 for 10–20 min, also at room temperature. Reactions were terminated by the addition of gel-loading buffer containing SDS and β-mercaptoethanol, followed by boiling for 2 min. Samples were analyzed by SDS-PAGE, followed by autoradiography.

In vivo labeling with ^{75}Se

Plasmids were transfected by $CaPO_4$ coprecipitation into HEK-293 cells as previously described (27). Three days before the transfection, these cells were plated onto 60-mm dishes in DMEM containing 10% FCS. One day before transfection, the medium was changed to DMEM containing 1% FCS supplemented with 100 pM T_3, 10 mg/ml transferrin, 20 μg/ml insulin, and 50 nM hydrocortisone to decrease the medium selenium concentrations. The day after transfection, the cells were shocked with 10% dimethylsulfoxide (2 min) and fresh medium containing 5 nM unlabeled Na_2SeO_3 plus 6 μCi Na_2[^{75}Se]O_3/dish. After 18 h of incubation, the cells were harvested, washed, resuspended in PE buffer, and sonicated. Aliquots of labeled cell sonicate were analyzed by SDS-PAGE.

Reagents

Bromoacetylchloride was obtained from Aldrich Chemical Co. (Rouses Point, IL). [^{125}I]T_4 and [^{125}I]T_3 were purchased from DuPont-New England Nuclear. [^{75}Se]Selenite was a generous gift from Dr. Dolph Hatfield (NIH) and was originally obtained from Dr. Kurt Zinn (University of Missouri Research Reactor, Columbia, MO). All other chemicals were of reagent grade.

Sequence analyses

Nucleotide and protein sequence analyses were performed using the Sequence Analysis Software Package from the Genetics Computer Group (University Research Park, Madison, WI).

Results

Sequence of the 5′-hD2 cDNA

A 2.1-kb partial cDNA clone (Z44085) was identified in a library derived from human infant brain prepared by Genethon by its significant identity (75%) with the 5′-region (1–173 nucleotides) of the *Rana* D2 cDNA (17) (St. Germain, D., and V. A. Galton, personal communication). The cDNA Z44085 contains an 822-bp open reading frame (nucleotides 40–861) that begins with an ATG codon at position 40–42, has two in-frame TGA codons at positions 436–438 and 835–837, and has a TAA codon at position 859–861 (Fig. 1). The deduced amino acid sequence predicts a protein of 273 residues, with a molecular mass of 30.5 kDa, assuming the two TGA codons encode selenocysteine. The deduced hD2 protein is similar to the *Rana* D2 (78% identity and 84% similarity) deduced amino acid sequence. The human enzyme is nine residues longer than *Rana* D2 (Fig. 2) and has three potential *N*-glycosylation sites (NXS) in the predicted sequence at residues 83, 100, and 261. Only one of these, at position 83, is conserved between the human and *Rana* proteins (Fig. 2).

A hydropathy analysis revealed a single hydrophobic amino-terminal portion consistent with a transmembrane domain, whereas the two selenocysteine residues are located in hydrophilic domains of the protein (Fig. 3). The amino-termini of both human and *Rana* D2 are highly hydrophobic and conserved.

Northern blotting

Previous studies have identified D2 activity in human brain and placenta (5, 12). A ^{32}P-labeled DNA containing the entire coding sequence of hD2 was used to probe a Northern blot containing 2 μg poly(A)$^+$ RNA from eight different human tissues. A prominent band of 7–8 kb was detected in the lanes corresponding to placenta, brain, and, surprisingly, heart and skeletal muscle (Fig. 4). This band was the primary

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1    CCAGAGCGCACAAGGGAACTGACTCAGGAGGCAGAGAAGATGGGCATCCTCAGCGTAGAC
                                          M  G  I  L  S  V  D

61   TTGCTGATCACACTGCAAATTCTGCCAGTTTTTTTCTCCAACTGCCTCTTCCTGGCTCTC
     L  L  I  T  L  Q  I  L  P  V  F  F  S  N  C  L  F  L  A  L

121  TATGACTCGGTCATTCTGCTCAAGCACGTGGTGCTGCTGTTGAGCCGCTCCAAGTCCACT
     Y  D  S  V  I  L  L  K  H  V  V  L  L  L  S  R  S  K  S  T

181  CGCGGAGAGTGGCGGCGCATGCTGACCTCAGAGGGACTGCGCTGCGTCTGGAAGAGCTTC
     R  G  E  W  R  R  M  L  T  S  E  G  L  R  C  V  W  K  S  F

241  CTCCTCGATGCCTACAAACAGGTGAAATTGGGTGAGGATGCCCCCAATTCCAGTGTGGTG
     L  L  D  A  Y  K  Q  V  K  L  G  E  D  A  P  N  S  S  V  V

301  CATGTCTCCAGTACAGAAGGAGGTGACAACAGTGGCAATGGTACCCAGGAGAAGATAGCT
     H  V  S  S  T  E  G  G  D  N  S  G  N  G  T  Q  E  K  I  A

361  GAGGGAGCCACATGCCACCTTCTTGACTTTGCCAGCCCTGAGCGCCCACTAGTGGTCAAC
     E  G  A  T  C  H  L  L  D  F  A  S  P  E  R  P  L  V  V  N

421  TTTGGCTCAGCCACTTGACCTCCTTTCACGAGCCAGCTGCCAGCCTTCCGCAAACTGGTG
     F  G  S  A  T  SeC  P  P  F  T  S  Q  L  P  A  F  R  K  L  V

481  GAAGAGTTCTCCTCAGTGGCTGACTTCCTGCTGGTCTACATTGATGAGGCTCATCCATCA
     E  E  F  S  S  V  A  D  F  L  L  V  Y  I  D  E  A  H  P  S

541  GATGGCTGGGCGATACCGGGGGACTCCTCTTTGTCTTTTGAGGTGAAGAAGCACCAGAAC
     D  G  W  A  I  P  G  D  S  S  L  S  F  E  V  K  K  H  Q  N

601  CAGGAAGATCGATGTGCAGCAGCCCAGCAGCTTCTGGAGCGTTTCTCCTTGCCGCCCCAG
     Q  E  D  R  C  A  A  A  Q  Q  L  L  E  R  F  S  L  P  P  Q

661  TGCCGAGTTGTGGCTGACCGCATGGACAATAACGCCAACATAGCTTACGGGGTAGCCTTT
     C  R  V  V  A  D  R  M  D  N  N  A  N  I  A  Y  G  V  A  F

721  GAACGTGTGTGCATTGTGCAGAGACAGAAAATTGCTTATCTGGGAGGAAAGGGCCCCTTC
     E  R  V  C  I  V  Q  R  Q  K  I  A  Y  L  G  G  K  G  P  F

781  TCCTACAACCTTCAAGAAGTCCGGCATTGGCTGGAGAAGAATTTCAGCAAGAGATGAAAG
     S  Y  N  L  Q  E  V  R  H  W  L  E  K  N  F  S  K  R  SeC  K

841  AAAACTAGATTAGCTGGTTAAAGGTATGATTATAAGAGAGCTTATTGTTTTAGCTTGATG
     K  T  R  L  A  G  *
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FIG. 1. The nucleotide and predicted amino acid sequences of the human D2 partial cDNA clones. The amino acid selenocysteine is noted as SeC.

signal detected in the blot after overnight exposure, but at longer intervals, smaller, less intense bands could also be detected in the positive lanes, possibly representing smaller transcripts. These do not correspond to the size of the D3 mRNA (~2.2 kb) we identified previously in this blot in placenta (20) or that of the hD1 mRNA (2 kb). We previously probed this blot with β-actin cDNA, confirming the presence of adequate quantities of well preserved mRNA in all lanes (20).

The partial hD2 cDNA requires a heterologous SECIS element to produce an active enzyme

We subcloned the entire 2.1-kb hD2 cDNA into an eukaryotic expression vector (CDM-8). HEK-293 cells transfected with this construct did not transiently express a functional D2 enzyme. We interpreted this result as indicating that the D2 SECIS element was not present in the approximately 1.2-kb 3′-untranslated region of this cDNA. To test this hypothesis we made a chimera in which the putative open reading frame of the hD2 cDNA (nucleotides 1–900) was cloned 5′ to the highly potent SECIS element of the hD3 selenoprotein in CDM-8 (20). Cells transfected with this construct produced a functional D2 enzyme based on kinetic analyses. To increase the transient hD2 expression further, we subcloned the PCR-generated Kozak-D2 5′ to the highly active SelP SECIS element (KD2-SelP) (22) into both CDM-8 and D10 expression vectors and transfected these into HEK-293 cells.

Kinetic studies

Cell sonicates from both D2-D3- and KD2-SelP (CDM-8 and D10)-transfected HEK-293 cells deiodinated the outer

```
 1 MGILSVDLLITLQILPVFFSNCLFLALYDSVILLKHVVLLLSRSKSTRGE 50
   ||:|||||||||||||||.|||||||||||||||:|:||||:| |.||||.:|:
 1 MGLLSVDLLITLQILPGFFSNCLFLALYDSVVLVKHVLLQLNRSKSSHGQ 50
   .    .    .    .    .    .    .
51 WRRMLTSEGLRCVWKSFLLDAYKQVKLGEDAPNSSVVHVSSTEGGDNSGN 100
   ||||||.|||||||.|||||||||||||:|||||.|:||    .:.||:.
51 WRRMLTPEGLRCVWNSFLLDAYKQVKLGGDAPNSNVIHV....TDKNSSS 96
   .    .    .    .    .    .    .
101 GTQEKIAEGATCHLLDFASPERPLVVNFGSATUPPFTSQLPAFRKLVEEF 150
    |..     |..|||||||.|||||||||||||||||.||||||.|:||||
97  GKP.....GTPCHLLDFASSERPLVVNFGSATUPPFISQLPAFSKMVEEF 141
    .    .    .    .    .    .    .
151 SSVADFLLVYIDEAHPSDGWAIPGDSSLSFEVKKHQNQEDRCAAAQQLLE 200
    |.|||||||||||||||||| || |  |:||||.|||||||||..|||
142 SAVADFLLVYIDEAHPSDGWAAPGIS..SYEVKKHRNQEDRCAAANKLLE 189
    .    .    .    .    .    .    .
201 RFSLPPQCRVVADRMDNNANIAYGVAFERVCIVQRQKIAYLGGKGPFSYN 250
    .:|||||.|||| ||||.| ||||.|||||||||||||.|||||||| ||
190 QYSLPPQCQVVADCMDNNTNAAYGVSFERVCIVQRQKIVYLGGKGPFFYN 239
    .    .    .    .    .    .    .
251 LQEVRHWLEKNFSKRUKKTRLAG*.. 274 HUMAN D2
    |||||:||| .|:|: .... :.
240 LQEVRQWLELTFGKKAESGQTGTEK* 265 RANA D2
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FIG. 2. Alignment of the predicted amino acid sequence of human and *Rana catesbeiana* D2 enzymes. Selenocysteine residues are abbreviated as U, and the stop codon is indicated by an *asterisk*.

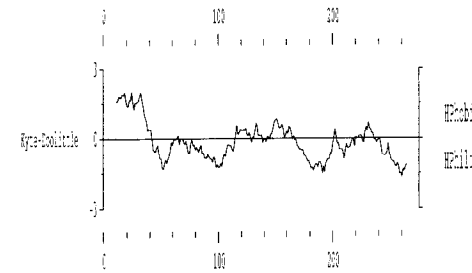

FIG. 3. Hydropathic analyses of human D2 protein using the Kyte-Doolittle algorithm (window = 21), as predicted by the PEPPLOT program of University of Wisconsin Genomic Computer Group.

ring of T_4, producing equimolar amounts of T_3 and I^-. The apparent K_m of the hD2 enzyme for T_4 was 4.0 nM, and the maximum velocity (V_{max}) was approximately 1.35 pmol I^- released/min·mg sonicate protein (Fig. 5 and Table 1). The reaction was stimulated by DTT, with maximal D2 activity observed at 20 mM (data not shown). The enzyme was relatively insensitive to PTU, with only about 25% inhibition observed at 2 mM PTU at varying DTT levels (Fig. 5C), and also to inhibition by T_3 (~30% inhibition at 3.0 μM T_3; Fig. 5D). The D2 enzyme was relatively insensitive to GTG, which is a noncompetitive inhibitor of T_4 5′-deiodination (K_i, ~1.0 μM; Fig. 5, A and B, and Table 1). rT_3 was also a good substrate for the D2 enzyme, with an apparent K_m of 9.1 nM and a V_{max} of 1.2 pmol I^- released/min·mg (Table I). rT_3 is a competitive inhibitor of T_4 deiodination by D2 (K_i, ~10 nM; data not shown).

Overexpressed hD2 is not covalently labeled during incubation with BrAc^{125}I T_3 or T_4

We attempted to covalently label the transiently expressed hD2 with $BrAcT_3$ or $BrAcT_4$. In some experiments, we increased the HEK-sonicate specific D2 activity by isolating the microsomal fraction (20,000–100,000 × *g* pellet) of transfected cell sonicates because D2 segregates with that fraction. When cells were transfected with the D10 vector alone and incubated with either $BrAcT_3$ or $BrAcT_4$, a number of labeled

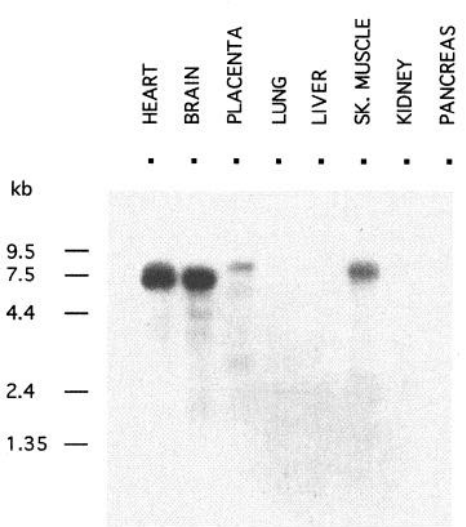

FIG. 4. Northern blot analysis of human D2 deiodinase. The blot was probed with a *Dra*I fragment containing the complete hD2 open reading frame. Each lane contains 2 μg human poly(A)$^+$ RNA. The same blot was previously hybridized with a mouse β-actin cDNA, which documented equal loading of each lane (20). The sizes of the mol wt markers are indicated.

bands were found, the most discrete of which were 64, 46, 34, and 15 kDa (Fig. 6). Sonicate protein from cells transfected with a rat D1 cDNA showed an additional ^{125}I-labeled band at 27–28 kDa corresponding to the D1 protein. Cells transfected with the KD2-SelP cDNA in the D10 vector in two different experiments (Fig. 6) did not show an additional labeled band. The V_{max} values for D2 activity in these preparations were 1.3 and 1.4 pmol I^- released/min·mg. An identical labeling pattern was observed when sonicates were preincubated for 10 min with 1 μM T_4 to block the D2 active site (Fig. 6), suggesting that there was no D2 protein comigrating with any of the bands labeled in the control sonicates. These experiments were repeated with three different sonicate preparations, all expressing more than 1 pmol I^- released/min·mg D2 activity with various modifications of the labeling conditions, but no additional labeled bands could be found.

^{75}Se labeling of the hD2 enzyme

Previous *in vivo* and *in vitro* studies using mammalian tissues suggested that D2 synthesis does not require selenium (28). However, both the *Rana* D2 and hD2 cDNAs contain in-frame TGA codons, presumably encoding selenocysteine. To confirm that the transiently expressed hD2 protein would incorporate selenium and to establish that the in-frame UGA codons at positions 436–438 and 835–837 do not function as stop codons, HEK-293 cells were transfected with the hD2 cDNA (hD2-SelP subcloned in both CDM and D10 vectors) and incubated with $Na_2[^{75}Se]O_3$ for 18 h. In the same experiments, we incubated cells transiently expressing the rat D1 protein with ^{75}Se as a positive control (Fig. 7). An approximately 31-kDa ^{75}Se-labeled doublet appeared after transfection with KD2-SelP (in both D10 and CDM) that was not present in the vector lanes (Fig. 7). The size of this doublet (~31 kDa) corresponds to that predicted by the deduced amino acid sequence of the D2 enzyme. The presence of the doublet indicates that two proteins differing by about 1 kDa are transiently expressed. This may occur due to premature termination of protein translation at the second UGA codon for some of the translation products.

D2 activity is present in human skeletal muscle

To prove that human skeletal muscle expresses the D2 protein, we prepared microsomes from a normal human skeletal muscle biopsy specimen obtained from the NCI Cooperative Human Tissue Network (Philadelphia, PA). Kinetic analyses revealed that skeletal muscle microsomes deiodinated T_4 with a K_m of 4.3 nM and a V_{max} of 0.01 pmol I^- released/min·mg (Fig. 8). This activity was not inhibited by 1 mM PTU.

Discussion

The present study describes the isolation and characterization of the transiently expressed human D2 enzyme. The deduced amino acid sequence shows 84% similarity to that of the recently reported *Rana* D2 (17) with an in-frame UGA codon in the same relative position. The UGA codon is essential for deiodinase activity during *in vitro* expression in *Xenopus* oocytes (17). Human and *Rana* D2 also contain a highly conserved hydrophobic amino-terminal segment of about 40 residues that presumably serves as a transmembrane domain based on the topological studies of a similar structure in the D1 enzyme (29). A similar hydrophobic NH_2-terminus is also present in human D3 (20).

However, there are some important differences between the human and *Rana* D2 mRNAs. The human D2 contains a second UGA codon near the carboxy-terminus of the protein, the first seleno-deiodinase in which a second in-frame UGA codon has been reported. Based on the presence of a ^{75}Se-labeled doublet in cell sonicates transiently expressing D2, it is possible that this UGA can serve as either a stop signal or a selenocysteine codon. It has recently been shown that the nucleotide after a UGA codon in selenoprotein mRNAs influences the specific function of this codon. A UGA codon followed by a pyrimidine is more likely to be translated as selenocysteine, whereas if it is followed by a purine, termination is favored (30). Consistent with this, the UGA codon in the active center of the D2 mRNA is followed by a C, whereas that in the carboxy-terminus is followed by an A. The predicted difference in size between a protein terminating at the second UGA and that at the terminal UAA is about 900 Da. This is the same difference as that seen between the two ^{75}Se-labeled bands. Further studies will be required to determine whether the truncated protein has properties different from those of the protein encoded by the full-length cDNA. Based on the fact that the *Rana* D2 functions well as a type 2 deiodinase despite the lack of identity in this region, it seems likely that the specific carboxy-terminal eight residues of hD2 are not essential for D2 activity.

A second major difference between the *Rana* and human D2 is in the size of the mRNA (7–8 kb for human and only 1.5 kb for *Rana*). The mRNAs for the human D1 and D3 enzymes are also relatively small (~2 kb) (2, 20). An addi-

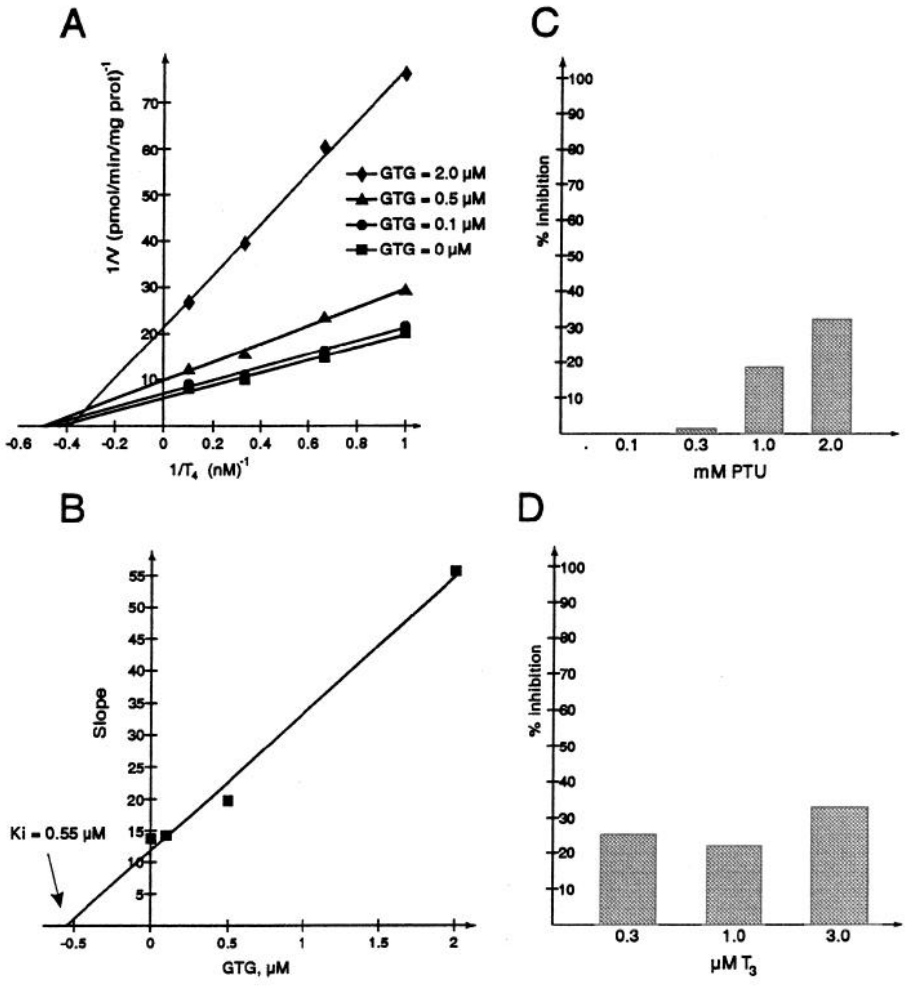

FIG. 5. Representative kinetic studies of the 5′-deiodination of T_4 by hD2 transiently expressed in HEK-293 cells. A, A Lineweaver-Burk plot of the 5′-deiodination of T_4 in the absence and presence of GTG. Reactions contained 2 nM T_4, 20 mM DTT, 1 mM PTU, and tracer [^{125}I]T_4 in 300 μl PE buffer, and incubations were performed for 60 min. B, Slope replot of the data in A. C and D, Effects of increasing concentrations of PTU (C) or T_3 (D) on T_4 5′-deiodination using the conditions described in A. Activities are expressed as either percent deiodination or velocity. Each *data point* is the average of closely agreeing duplicate determinations.

TABLE 1. Kinetics of 5′ deiodination by transiently expressed hD2

Exp	K_m (T_4) nM	V_{max} (T_4) pmol/min/mg prot	K_m (rT_3) nM	V_{max} (rT_3) pmol/min/mg prot	K_i GTG (T_4) μM
1	5.4	1.3	9.1	1.1	0.6
2	4.0	1.4	7.1	1.2	2.1

tional difference is that in the *Rana* D2, a SECIS element is found within the first 600 nucleotides of the 3′-untranslated region, whereas the partial hD2 2.1-kb cDNA clone in Z44085 used in these studies contains no functional SECIS activity (17). Expression of hD2 required a heterologous SECIS element from hD3 or SelP. The location of the SECIS element in the hD2 mRNA remains to be determined.

Previous studies have shown that D2 activity is present in human placenta, anterior pituitary, central nervous system, keratinocytes, and BAT (7, 9, 12, 13, 31, 32). However, the Northern blot indicates that not only is this message expressed in human placenta and cerebral cortex, as expected, it is found also in cardiac and skeletal muscle. This is completely unexpected, but, indeed, we found that D2 activity was present in human skeletal muscle at levels similar to those reported in human keratinocytes and placenta (13). D2 activity has also been found in tadpole tail (33), and T_4 to T_3 conversion has been demonstrated *in vivo* in perfused rat hindlimb (34). Although D2 *per se* has not previously been detected in mammalian skeletal muscle, even at relatively low levels it could have considerable significance in peripheral T_3 production due to the large mass of this tissue. T_3 produced from T_4 catalyzed by D2 would not be decreased by PTU treatment, and such a source could account for the PTU-insensitive T_3 production in humans demonstrated in *in vivo* studies (35). Supporting this are earlier kinetic results suggesting that about 50% of peripheral T_4 to T_3 conversion in rats derives from a slowly equilibrating T_4 pool that contains skeletal muscle (36). Further studies of cardiac and skeletal muscle will be required to estimate the potential contributions of these organs to peripheral T_3 production.

The kinetic studies demonstrate that the apparent K_m values of the transiently expressed enzyme for T_4 and rT_3 are identical to those previously reported for human placenta and rat BAT microsomes (12, 37). The transiently expressed D2 activity (~1 pmol I^- released/min·mg) is about 10 times

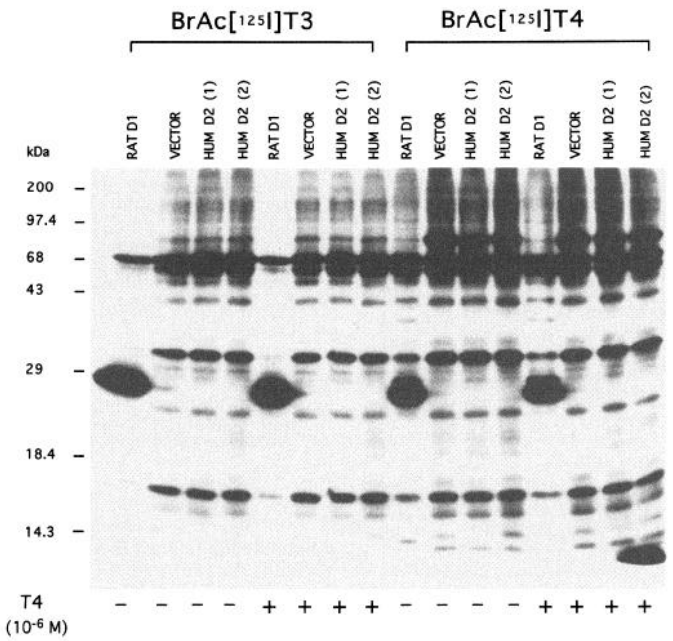

FIG. 6. $BrAcT_3$ and $BrAcT_4$ labeling of microsomal fractions from two different preparations of HEK-293 cell sonicates transiently transfected with an hD2-expressing D2 [HUM D2 (1) and (2)] in the D10 vector. Microsomal protein (120 μg) was incubated without (−) or with (+) 1×10^{-6} M T_4 for 10 min before a 10-min incubation at room temperature with $BrAc[^{125}I]T_3$ or -T_4 (0.1 pmol). Control lanes contained transiently expressed rat D1 (RAT D1) or microsomes from HEK-293 cells transfected with empty vector (D10). Reactions were stopped with gel-loading buffer and analyzed by SDS-PAGE. The hD2 activities (V_{max}) of the microsomal protein were 1.3 and 1.4 pmol I^- released/min·mg for microsomal preparations 1 and 2, respectively.

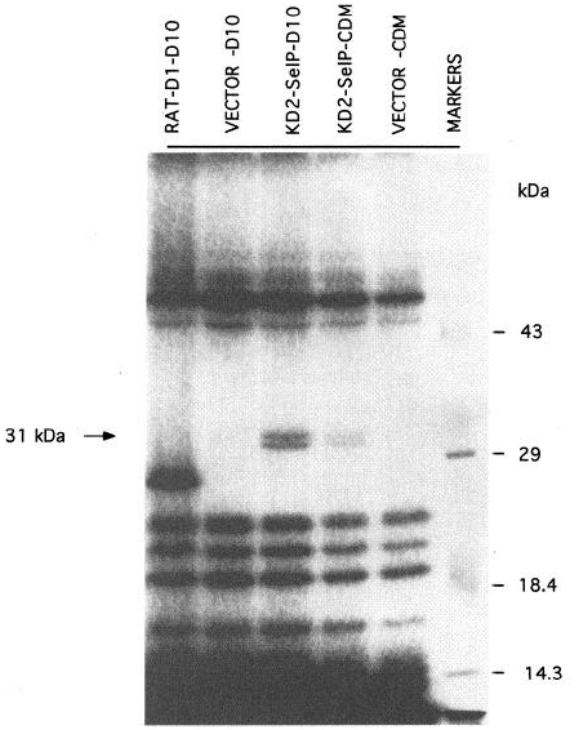

FIG. 7. Incorporation of ^{75}Se into transiently expressed human D2 protein. HEK-293 cells were incubated in selenium-depleted medium for 24 h as described in *Materials and Methods* before transfection of two different plasmids expressing the hD2 protein (KD2-SelP CDM and KD2-SelP D10). In the same experiment, a rat D1 protein expressing plasmid was also transfected as a positive control.

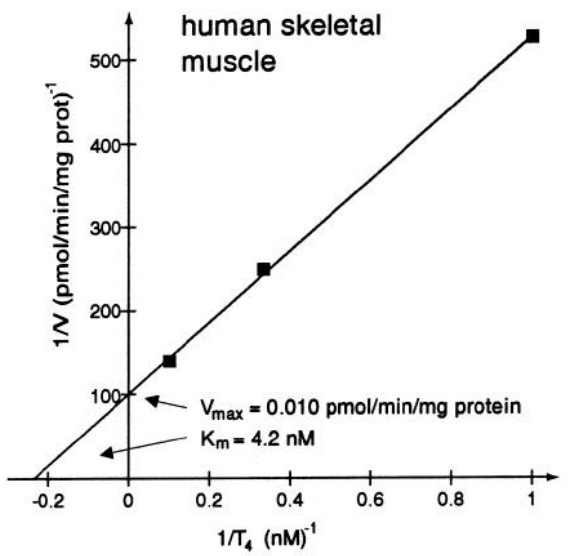

FIG. 8. Lineweaver-Burk plot of T_4 deiodination by human skeletal muscle microsomes. Reactions contained 20 mM DTT in 300 μl PE buffer, 90 μg microsomal protein, 1 mM PTU, and various concentrations of T_4. Incubations were performed for 2 h at 37 C.

higher than that in BAT microsomes from cold-exposed rats (0.08 pmol I^- released/min·mg) (37). In addition, catalysis by D2 is relatively insensitive to PTU, with only 20–30% inhibition at 2 mM. This contrasts with outer ring deiodination by the D1 enzyme, which is completely inhibited at micromolar PTU concentrations (2). We previously reported that T_4 5′ deiodination by rat D2 from BAT was competitively inhibited by GTG (7). The apparent K_i values for GTG in BAT were approximately 0.8 and 2.4 μM, quite similar to the K_i of 2 μM we found for inhibition of hD2. However, the kinetic analyses of inhibition of hD2 activity by GTG in the present studies suggest that this occurs by noncompetitive, rather than competitive, inhibition. The K_i of 1–2 μM for GTG contrasts with the K_i of 3.2 nM for competitive GTG inhibition of D1 activity (7). As expected, although rT_3 is a potent competitive inhibitor of T_4 deiodination by D2, T_3 has little effect on this process.

Despite the fact that D2, like D1, is a selenoenzyme, neither *in vivo* nor *in vitro* selenium deprivation reduces D2 activities, as occurs with D1 (38–40). Although initially these results were used as evidence that D2 was not a selenoenzyme, they must have other explanations, such as preferential utilization of selenocysteine for D2 synthesis or the fact that there is a very limited requirement for selenocysteine due to the low number of D2 enzyme molecules present. Likewise, in earlier studies, we found that substitution of cysteine for selenocysteine in D1 markedly reduced the sensitivities of the reaction to PTU and GTG, making it resemble D2. This again suggested that D2 might have a cysteine, not a selenocysteine, in its active center (1). Subsequent studies using the dog D1 or a Phe^{65}-Leu mutant human D1, both of which have much lower Kcat values for rT_3 than the wild-type human or

rat D1 enzymes, showed that even when selenocysteine is present in the active center, sensitivities to PTU and GTG were significantly reduced (3). This indicated that a low rate of enzyme turnover reduced sensitivity to PTU and GTG even though selenocysteine is present in the active site (3). If hD2, like hD3, has a low turnover rate, it would account for its insensitivity to PTU and GTG (20). As we have not been able to covalently label the transiently expressed enzyme with $BrAcT_3$ or T_4, we cannot calculate the Kcat to confirm this hypothesis. That the hD2 does contain selenocysteine is shown in the studies in which two ^{75}Se-labeled proteins of approximately 30 and 31 kDa are produced in cells transfected with an expression vector containing the hD2-coding sequence, but not in control cells. Thus, insensitivity to selenium deprivation, PTU, or GTG is not a specific criterion indicating the absence of selenocysteine in the active center of a selenodeiodinase.

Our inability to label the transiently expressed D2 protein contrasts with reports of others using the cAMP-stimulated astroglial cell model (40). These researchers reported $BrAcT_4$-labeled proteins of 55, 27, and 18 kDa in unstimulated astroglia, with a 5-fold increase in the 27-kDa protein after $(Bu)_2$cAMP exposure, in association with parallel increases in D2 activity up to 0.06 pmol I^- released/min·mg protein (40). Although the activities of the transiently expressed D2 in the preparations we used (1 pmol I^- released/min·mg) were at least 10-fold higher than those in stimulated astroglial cells, we could not identify any specific labeled bands after exposure to either $BrAcT_3$ or $BrAcT_4$. We also noted no decreases in the density of any band after prior exposure to 1 μM T_4, suggesting that there is no D2 protein comigrating with any of the bands that are labeled by $BrAcT_3$ or $BrAcT_4$ in the control sonicates. In the same experiments, rat D1 was readily labeled. We are not able to explain these discrepancies, although they could be due to differences between the rat and human D2 proteins or to the cells used. Nonetheless, our results show that, as with other characteristics, such as sensitivity to inhibition by PTU and GTG, which were once thought to be an intrinsic characteristic of a selenodeiodinase, the capacity to bind $BrAcT_3$ or $BrAcT_4$ is markedly influenced by factors other than the presence of selenocysteine in the active center.

In conclusion, the present studies reveal unexpected complexities in the human D2 that make it quite different from the other two human selenodeiodinases and from the *Rana* D2. The large size of the mRNA, the different and still unknown location of the SECIS element, and the presence of the D2 mRNA in skeletal and cardiac muscle, are all intriguing new observations indicating that the contributions of this enzyme to human thyroid physiology may be even more important than previously recognized.

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