Deubiquitination of type 2 iodothyronine deiodinase by von Hippel–Lindau protein–interacting deubiquitinating enzymes regulates thyroid hormone activation

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The type 2 iodothyronine deiodinase (D2) is an integral membrane ER-resident selenoenzyme that activates the pro-hormone thyroxine (T4) and supplies most of the 3,5,3′-triiodothyronine (T3) that is essential for brain development. D2 is inactivated by selective conjugation to ubiquitin, a process accelerated by T4 catalysis and essential for the maintenance of T3 homeostasis. A yeast two-hybrid screen of a human-brain library with D2 as bait identified von Hippel–Lindau protein–interacting deubiquitinating enzyme-1 (VDU1). D2 interaction with VDU1 and VDU2, a closely related deubiquitinase, was confirmed in mammalian cells. Both VDU proteins colocalize with D2 in the ER, and their coexpression prolongs D2 half-life and activity by D2 deubiquitination. VDU1, but not VDU2, is markedly increased in brown adipocytes by norepinephrine or cold exposure, further amplifying the increase in D2 activity that results from catecholamine-stimulated de novo synthesis. Thus, deubiquitination regulates the supply of active thyroid hormone to brown adipocytes and other D2-expressing cells.

Methods

Yeast two-hybrid screening. The yeast two-hybrid Matchmaker 3 system (CLONTECH Laboratories Inc., Palo Alto, CA) was used to isolate VDU1. The COOH-terminal portion of human D2 was used as bait in a yeast two-hybrid system. D2 interaction with VDU1 and VDU2, closely related deubiquitinases, was confirmed in mammalian cells. Both VDU proteins colocalize with D2 in the ER, and coexpression prolongs D2 half-life and activity by D2 deubiquitination. VDU1, but not VDU2, is markedly increased in brown adipocytes by norepinephrine or cold exposure, further amplifying the increase in D2 activity that results from catecholamine-stimulated de novo synthesis. Thus, deubiquitination regulates the supply of active thyroid hormone to brown adipocytes and other D2-expressing cells.

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Nonstandard abbreviations used: thyroxine (T4); 3,5,3′-triiodothyronine (T3); types 1 and 2 iodothyronine deiodinase (D1 and D2); brown adipose tissue (BAT); ubiquitinated D2 (UB-D2); ER-associated degradation (ERAD); von Hippel–Lindau protein (pVHL); pVHL-interacting deubiquitinating enzyme (VDU); GAL4 activation domain (GAL4 AD); hemaglutinin (HA); GAL4 DNA binding domain (GAL4 DBD); quadruple dropout (QDO); wild-type D2 (wtD2); glutathione-S-transferase (GST); immunoprecipitation (IP); norepinephrine (NE); ubiquitin-specific processing protease (UBP).

Conversion of T4 to T3 by D2 accelerates the ubiquitination of D2, inactivating it and thus serving as an important posttranslational feedback mechanism for maintaining T3 homeostasis (2-5). While the inactive ubiquitinated D2 (Ub-D2) is subject to ER-associated degradation (ERAD), our present studies demonstrate that Ub-D2 is the first recognized substrate for the recently discovered von Hippel–Lindau protein–interacting deubiquitinating enzyme-1 (VDU1) (6). VDU1 was isolated from human brain using the COOH-terminal portion of human D2 as bait in a yeast two-hybrid system. The interaction of D2 with VDU1 and VDU2, closely related deubiquitinases, was confirmed in mammalian cells. Both VDU proteins colocalize with D2 in the ER, and their coexpression prolongs D2 half-life and activity by D2 deubiquitination. VDU1, but not VDU2, is markedly increased in brown adipocytes by norepinephrine or cold exposure, further amplifying the increase in D2 activity that results from catecholamine-stimulated de novo synthesis. Thus, deubiquitination regulates the supply of active thyroid hormone to brown adipocytes and other D2-expressing cells.

Alto, California, USA) was used to screen for potential D2-interacting proteins. A human-brain cDNA library cloned into pACT2 such that it contains a GAL4 activation domain (GAL4 AD) and an hemaglutinin (HA) tag amino-terminal was pretransformed into the yeast strain Y187. This library was then screened by mating with AH109 yeast containing the D2 bait construct pGBKKT7-D2. pGBKKT7-D2 encodes a fusion protein that contains amino acids 1–147 of the GAL4 DNA binding domain (GAL4 DBD), a myc tag, and then amino acids 166–273 of human D2, with the selenocysteine at 266 mutated to cysteine. The D2 fragment was prepared by PCR amplification of a clone of human D2. This PCR product was digested and cloned in NcoI/EcoRI sites of pGBKKT7, and the resulting plasmid was confirmed by sequencing. Western blot analysis using anti-myc antibody (CLONTECH Laboratories Inc.) further confirmed pGBKKT7-D2 expression in AH109. According to the manufacturer’s instructions, yeast were initially selected on triple-dropout media that lacked the vector-selective markers leucine and tryptophan, as well as histidine; then they were re-streaked on the most stringent selection media, i.e., quadruple-dropout (QDO) media that lacked leucine, tryptophan, histidine, and adenine and contained 20 µg/ml X-α-gal, allowing for blue/white selection. Single colonies were picked and then re-streaked on QDO media containing X-α-gal two more times. Yeast colonies were then grown up, and DNA was extracted by lyticase digestion followed by detergent lysis. Purified DNA was transformed back into Escherichia coli and isolated by Qiaprep Spin Miniprep Kit (Qiagen, Valencia, California, USA). Each clone was amplified by PCR using PCR Platinum SuperMix (Invitrogen Life Technologies, Carlsbad, California, USA) and pACT2-specific primers flanking the region of the library insert to confirm a single PCR product, hence one unique clone. This DNA was reintroduced into the yeast strain Y187 and mated with yeast AH109 that contained either pGBKKT7-D2 or, as a control for nonspecific interactions, empty pGBKKT7 expressing only the GAL4 DBD. Clones that grew appropriately on QDO media containing X-α-gal and were determined not to interact with the GAL4 DBD alone were sequenced and blasted against the GenBank database. Since these clones are expressed with the GAL4 AD fused amino-terminally, only clones determined to be in the same reading frame as the GAL4 AD were pursued. The interaction between D2 and VDU1 was confirmed by mating of yeast that contained pGBKKT7-D2 with yeast that contained pGADT7-VDU1. The resulting yeast cells grew on QDO media, whereas the negative and positive controls behaved as expected (Figure 1b).

Plasmids, transfections, and D2 activity. HEK-293 cells were transfected by the CaPO 4 method (3) with plasmids containing human wild-type D2 (wtD2) (10), FLAG-CysD2 in which the Sec133 was replaced by Cys (4), FLAG-wtD2 (11), GFP-VDU1 (6), GFP-VDU2 (7), wild-type D1 (wtD1) (12), or empty vector (D10). In all experiments, human growth hormone (thymidine kinase growth hormone) was used as a control for the transfection efficiency, as described previously (13). For the VDU-D2 interaction studies (Figure 1, b and c), immunofluorescence (Figure 2b), and identification of HA-Ub-D2 (Figure 3e), a catalytically active CysD2 was used, because Sec-encoding mRNAs are translated at a much lower rate (20- to 400-fold) than non-Sec-encoding mRNAs (14). We have previously demonstrated that both wild-type CysD2 and transiently expressed CysD2 in HEK-293 cells are targeted by the ubiquitin-proteasome system (3). For all other experiments, either wtD2 or FLAG-wtD2 was transiently expressed in HEK-293 cells. Because of the small amounts of plasmid DNA that were used for transfection (1–5 µg), D2 activity in these cells was not higher than in a human mesothelioma cell line that endogenously expresses D2 (15).

Cloning of Flag-wtD2. wtD2 was transiently expressed in HEK-293 cells. Because of the small amounts of plasmid DNA that were used for transfection (1–5 µg), D2 activity in these cells was not higher than in a human mesothelioma cell line that endogenously expresses D2 (15).

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Figure 1

Yeast two-hybrid mating for pGBK7-T-D2 and VDU1-expressing clone. (a and b) Positive control interaction for the mating: AH109 (pGBK7-T53) plus Y187 (pTD1); negative control for the mating: AH109 (pGBK7-T-empty) plus Y187 (pGADT7-empty); mating: AH109 (pGBK7-T2) plus Y187 (pGADT7-VDU1); negative control for the VDU1 mating: AH109 (pGBK7-empty) plus Y187 (pGADT7-VDU1). (c) Mating grown on Trp-Leu double-dropout (DDO) media. (b) Mating grown on Trp-Leu-His-Ade-α-Gal media plus X-α-Gal substrate. While all yeast strains grew on DDO media, indicating the presence of both expression plasmids, D2 did not interact with the GAL4 AD alone (data not shown), and, on QDO media (b), VDU1 did not interact with the GAL4 DBD alone, while pGBK7-T-D2 interacted with the pGADT7-VDU1 clone. The top left panel indicates the growth of the positive control, while the negative control is indicated in the top right panel. The growth of pGBK7-D2 + VDU-1 on QDO media plus X-α-Gal is shown on the lower left panel, while pGBK7-empty + VDU-1 is shown on the lower right panel, indicating specificity of VDU-1 interaction with D2. (c) In vitro coimmunoprecipitation of GST-D2 and 35S-VDU1. Crude bacterial lysates expressing GST-D2 or GST-GUS were incubated with 35S-VDU1 followed by GST pulldown. Pellets were resolved by SDS-PAGE, and 35S-VDU1 is indicated. Levels of GST-fusion proteins (GST-D2 and GST-GUS) were determined by Western analysis using anti-GST antibody. Approximately 1% of the input 35S-VDU1 is specifically pulled down. This experiment was performed twice. (d) In vivo coimmunoprecipitation of D2 and VDU1 or VDU2. HEK-293 cells were cotransfected with FLAG-CysD2 and GFP-VDU1 or GFP-VDU2. Anti-GFP antibody was used for immunoprecipitation, and the pellets were resolved by SDS-PAGE and probed with anti-GFP antibody by Western analysis. GFP refers to a vector containing only GFP, not fused to any other protein. This experiment was performed twice with similar results.

Previously described (11). Pellets were resolved in a 10% SDS-PAGE gel, dried, and exposed to autoradiography for 35S-labeled band detection. For the in vivo coimmunoprecipitation experiments, HEK-293 cells transiently expressing 5 μg GFP-VDU1 or 5 μg GFP-VDU2 and 5 μg FLAG-CysD2 were resuspended in lysis buffer containing 1× PBS, 5 mM EDTA, 0.5% Triton X-100 (Sigma-Aldrich), type I protease inhibitor cocktail (Calbiochem-Novabiochem Corp., San Diego, California, USA), and 1 mM PMSF. Cell lysates with each transfection combination were incubated with Living Colors Full-Length Av polyclonal antibody (anti-GFP antibody; BD Biosciences Clontech, Palo Alto, California, USA) at a dilution of 1:300. Forty microliters of Protein G PLUS/Protein A-Agarose bead suspension (Oncogene Research Products, San Diego, California, USA) was added, and IP pellets were washed five times with 1× PBS, resuspended in sample loading buffer, boiled for 5 minutes, resolved in a 10% SDS-PAGE, and used for Western blot analysis for FLAG-CysD2 detection. FLAG-CysD2 detection, using anti-FLAG M2 mAb (Sigma-Aldrich) at a dilution of 1:2,000 (4), was performed using the BM chemiluminescence Western blotting kit (Roche, Indianapolis, Indiana, USA) according to the manufacturer’s instructions.

In vivo D2 deubiquitination. HEK-293 cells transiently expressing 5 μg FLAG-CysD2 and 5 μg HA-Ub, with or without 5 μg VDU1 or 5 μg VDU2, were processed for IP as described above using anti-FLAG antibody at 1:250. The IP pellets were resolved in a 7.5% SDS-PAGE gel and used for Western blot analysis with anti-HA polyclonal antibody (Clontech Laboratories Inc.).

Immunofluorescence confocal microscopy. Immunofluorescence confocal microscopy was done as described previously (16) with minor modifications. HEK-293 cells were transfected with GFP-VDU1 or GFP-VDU2 and FLAG-CysD2. After parafomaldehyde fixation, cells were permeabilized with 0.25% Triton X-100 and incubated with anti-FLAG antibody at a dilution of 1:500. This was followed by 30 minutes’ incubation with affinity-purified goat anti-mouse Texas red F(ab’)2; fragment previously described (11). Pe...
male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine, USA) weighing 20–30 g were kept in individual cages on a 12-hour/12-hour dark/light cycle with free access to chow and water. Animals were maintained at room temperature or exposed to 4°C for up to 48 hours, as indicated in the figures. As indicated, animals were injected with 0.75 µg norepinephrine (NE) per 1 g body weight. Animals were euthanized, and the tissues were immediately isolated and frozen in liquid nitrogen for RNA isolation and/or cell lysis for subsequent deiodinase assay.

**Real-time PCR.** Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and used to synthesize cDNA using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies). The generated cDNAs were used in a real-time PCR using the QuantiTect SYBR Green PCR kit in I-Cycler (Bio-Rad). Standard curves representing five-point serial dilution of mixed cDNA of the experimental and control groups were analyzed in each assay and used as calibrators to the relative quantification of product generated in the exponential phase of the amplification curve. The r² was greater than 0.99 for all standard curves, and the amplification efficiency varied between 80% and 100%. Sample quantification was calculated by the standard curve and corrected by the internal control β-actin mRNA in all experiments. Specific oligonucleotides for mouse VDU1, VDU2, D2, and β-actin were designed using Beacon Designer 2.06 (PREMIER Biosoft International, Palo Alto, California, USA).

**Results**

**VDU1 and VDU2 interact specifically with D2 in vivo and colocalize in the ER.** We screened a human-brain library with a C-terminal fragment of human D2 (residues 166–273) using a yeast two-hybrid system and identified a specific interacting protein containing the amino acids 585–786 of the recently identified VDU1 (GenBank accession no. AF383173) (Figure 1, a and b). Using GST pulldown, we verified that the interaction between GST-D2 and 35S-VDU1 occurs in vitro (Figure 1c). Moreover, in HEK-293 cells, we demonstrated that D2 interacts not only with VDU1 but also with the closely related VDU2, as FLAG-CysD2 is coimmunoprecipitated with either GFP-VDU1 or GFP-VDU2, but not in vector-transfected cells (Figure 1d).
Subcellular fractionation of HEK-293 cells transiently expressing VDU1 and VDU2 revealed that both proteins are found in the particulate pellet (microsomes) but not in the cytosolic fraction (Figure 2a). Analysis of the protein sequences suggests a low probability of a single transmembrane domain (17), suggesting that VDUs are membrane-associated, rather than integral membrane, proteins.

Immunofluorescence confocal microscopy studies of HEK-293 cells transiently coexpressing FLAG-CysD2 and GFP-VDU1 or FLAG-CysD2 and GFP-VDU2 indicate that both VDU1 and VDU2 colocalize with D2 in the perinuclear region (Figure 2b). Furthermore, VDU1 and VDU2 also colocalize with BIP, an ER-resident chaperone (data not shown).

D2 is a substrate for VDU1 and VDU2. Coexpression of VDU1 or VDU2 with D2 in HEK-293 cells increased basal D2 activity 1.7- and 2.1-fold, respectively (Figure 2c). Protein synthesis was blocked with cycloheximide to ascertain whether coexpression with VDU1 or VDU2 stabilizes D2 activity. Blockade of protein synthesis for 2 hours reduced D2 activity to about 25% of that in vehicle-treated cells, which is compatible with previous observations (3). However, D2 activity remained stable after protein synthesis was blocked in cells coexpressing either VDU1 or VDU2, indicating a marked increase in the half-life of active D2 (Figure 2d).

Deubiquitination of D2 would be predicted to decrease its uptake by proteasomes, which is known to be rate limiting in D2 degradation (2, 3). This was demonstrated in HEK-293 cells coexpressing FLAG-D2 and VDU1 or VDU2 labeled with Na2[75Se]O3 and pulse-chased with 100 nM Na2SeO3. Pulse-chase studies revealed that 75Se-D2 protein decreases about 70% within 2 hours (Figure 3a), which is compatible with a 30-minute half-life. When coexpressed with VDU1 or VDU2 in a large series of experiments, the rate of decrease in 75Se-D2 is reduced to 15–40% within 2 hours, confirming that both VDUs stabilize D2 protein. This effect is specific, since the levels of 75Se-D1, an isoenzyme that is not ubiquitinated, were not affected by coexpression with VDU1 or VDU2 (Figure 3b).

To confirm that it is deubiquitination by VDU1 and VDU2 that stabilizes D2, lysates of HEK-293 cells transiently expressing FLAG-CysD2, HA-Ub, and VDU1 or VDU2 were lysed and processed for immunoprecipitation with anti-FLAG antibody. The pellets were resolved by SDS-PAGE and probed with anti-HA antibody by Western analysis. HEK-293 cells transiently coexpressing FLAG-wtD2 and/or VDU1 or VDU2 were labeled with 75Se for 16 hours, and the cell lysates were processed as described in Figure 3a. The corresponding immunoprecipitated nonubiquitinated 75Se-D2 is shown below each lane of 75Se-Ub-D2. Each experiment in a-c was performed twice.
4°C for 1–48 hours. After 6 hours, the expected approximately tenfold increase in D2 mRNA levels occurred (Figure 4a), but D2 activity was increased to a much greater extent, approximately 26-fold. Remarkably, VDU1 mRNA was increased approximately fourfold in BAT within 4 hours of cold exposure, an effect that is mimicked by NE injection in mice at room temperature (Figure 4b). Interestingly, VDU2 mRNA levels were not increased in brown adipocytes of cold-exposed mice, indicating that this autonomic response is VDU subtype–specific.

Discussion
The present studies identify D2 as the only known specific substrate of VDU1 and VDU2, which in turn are the first ubiquitin-specific processing proteases (UBPs) known to specifically deubiquitinate an ERAD substrate. These results show that protein recognition is involved not only in the E3-mediated ubiquitination process but also in the deubiquitination pathway catalyzed by UBPs. Both VDUs are downstream targets for ubiquitination by pVHL E3 ligase, and VHL mutations that disrupt the interaction between the VDUs and pVHL abrogate their ubiquitination (6, 7). Although hundreds of UBP enzymes have been cloned, only a few examples of substrate recognition by UBP enzymes have been

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**Figure 4**
Sympathetic stimulation markedly induces VDU1 expression in brown adipocytes. (a) Mice were exposed to cold for the indicated times. D2 mRNA levels in the BAT were measured by real-time PCR. β-Actin mRNA was used as internal control, and the results are expressed as fold change in the mRNA ratio compared with that of controls. D2 activity is expressed as fold change versus controls. (b) VDU1 and VDU2 mRNA levels were measured as in a. Experimental animals were treated with 0.75 µg NE per 1 g body weight, and control animals received saline. Values are the mean ± SD of two to four animals. The entire experiment was performed twice.

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**Figure 5**
Proposed model for reversible D2 ubiquitination. D2 is an ER-resident selenoprotein that is inactivated by selective ubiquitination via a process that is accelerated by catalysis of T4 deiodination (step 1). D2 ubiquitination involves a ubiquitin-activating enzyme (E1) (4), a ubiquitin conjugase (UBC7) (5, 31), and an as-yet unidentified ubiquitin ligase (E3) (step 2). Ub-D2 can either be taken up by the proteasome and irreversibly degraded (step 3) or interact with (step 4) and be reactivated by (step 5) VDU1- or VDU2-mediated deubiquitination.
reported, and, to our knowledge, none are ER-resident proteins (18–22).

VDU1 specifically binds to D2 in vitro (Figure 1c), as well as in yeast (Figure 1, a and b) and mammalian cells (Figure 1d). Since only the COOH-terminal portion of D2 (amino acids 166–273) was used as bait in the yeast two-hybrid screening, the VDU1 binding site is localized to this region of D2, which is predicted to be in the cytosol (16). There is no apparent homology between D2 and pVHL (23, 24), to which VDU1 binds specifically and of which it is a substrate, suggesting that different VDU1 binding sites are involved when it interacts with a substrate or pVHL. Our confocal studies (Figure 2b) indicate that both VDUs colocalize with D2, itself an integral membrane ER-resident protein. Although they are present in the particulate fraction and not in the cytosol (Figure 2a), it is not clear based on their hydrophobic profile whether VDU1 and VDU2 are integral membrane proteins. Their physical colocalization with D2, however, provides the opportunity for catalysis and D2 deubiquitination (Figure 2b).

D2 contains the rare amino acid selenocysteine in its active center. Decoding of the selenocysteine-encoding UGA codon to signal for cotranslational insertion of selenocysteine, not termination, in selenoproteins such as D2 decreases translation efficiency 20- to 400-fold (14). The transient expression of wtD2 in HEK cells results in D2 activities that are similar to those in a human mesothelioma cell line, MSTO211H (15). This argues for the physiological relevance of the observation that transient coexpression of either UBP in D2-expressing HEK-293 cells increases D2 activity, as would be expected if D2 is a specific VDU substrate. This observation is based on stabilization of both D2 activity (Figure 2, c and d) and 75Se-labeled protein levels (Figure 3a), in agreement with the finding of decreased levels of Ub-D2 during coexpression with VDU1 or VDU2 (Figure 3c). These data indicate that VDU1- or VDU2-catalyzed deubiquitination recycles inactive Ub-D2 to its active deubiquitinated form, bypassing the proteasomal degradation pathway. Thus, Ub-D2 can be either reactivated or degraded, with the balance between these two processes determined, at least in part, by the VDUs’ activities (Figure 5).

The present study also provides evidence that VDU1-catalyzed D2 deubiquitination is an important part of the adaptive mechanism that regulates thyroid hormone action. In stimulated brown adipocytes, normal D2 induction increases intracellular T3 production, resulting in isolated tissue thyrotoxicosis (9). This is an important mechanism for cold acclimatization, in that mice with targeted inactivation of the D2 gene develop hypothermia and marked weight loss during cold exposure due to impaired BAT thermogenesis (25). Our results demonstrate that increased VDU1-catalyzed deubiquitination of a pool of Ub-D2 and its rescue from proteasomal degradation are an integral part of this mechanism. VDU1 mRNA levels are markedly upregulated by cold exposure or NE (Figure 4), amplifying the transcriptional increase in D2 activity and hence T3 production approximately 2.5-fold (Figure 4a). Even though UBP induction is known to play a physiological role in a number of cellular processes (26–30), to our knowledge this is the first example of enzyme reactivation due to deubiquitination.

Thus, due to the intrinsic inefficiency of the selenoprotein synthesis, the availability of a reversible ubiquitination-dependent mechanism to control the activity of D2 constitutes a biochemical and physiological advantage that allows for rapid control of thyroid hormone activation (Figure 5). The finding that VDU1 and VDU2 are coexpressed with D2 in many human tissues, including brain, heart, and skeletal muscle (1, 6), indicates that the importance of this mechanism may extend well beyond thermal homeostasis to include brain development, cardiac performance, glucose utilization, and energy expenditure.

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