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Triplets! Unexpected Structural Similarity Among the Three Enzymes That Catalyze Initiation and Termination of Thyroid Hormone Effects

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ABSTRACT

The three iodothyronine deiodinases catalyze the initiation (D1, D2) and termination (D3) of thyroid hormone effects in vertebrates. A recently conceived 3-dimensional model predicts that these enzymes share a similar structural organization and belong to the thioredoxin (TRX) fold superfamily. Their active center is a selenocysteine-containing pocket defined by the $\beta 1$-$\alpha 1$-$\beta 2$ motifs of the TRX fold and a domain that shares strong similarities with the active site of iduronidase, a member of the clan GH-A fold of glycoside hydrolases. While D1 and D3 are long-lived plasma membrane proteins, D2 is an endoplasmic reticulum resident protein with a half-life of only 20min. D2 inactivation is mediated by selective UBC-7-mediated conjugation to ubiquitin, a process that is accelerated by T_4 catalysis, thus maintaining local T_3 homeostasis. In addition, D2 interacts with and is a substrate of the pVHL-interacting deubiquitinating enzymes (VDU1 and VDU2); thus deubiquitination regulates the supply of active thyroid hormone in D2-expressing cells. **(Arq Bras Endocrinol Metab 2004;48/1:16-24)**

Keywords: Deiodinases; Thyroid hormones; Selenocysteine; T_3 homeostasis

RESUMO

Trigêmeas! As Três Enzimas que Catalisam a Iniciação e Terminação dos Efeitos dos Hormônios Tiroideanos Exibem Semelhança Estrutural Inesperada.

As três desiodases de iodotironinas catalisam a iniciação (D1, D2) e o término (D3) dos efeitos dos hormônios tiroideanos em vertebrados. Um modelo tridimensional concebido recentemente propõe que essas enzimas apresentam organização estrutural similar e pertençam à superfamília da *thioredoxin (TRX)-fold*. O sítio ativo é formado por um bolso contendo selenocisteína, definido pelos motivos $\beta 1$-$\alpha 1$-$\beta 2$ do *TRX-fold*, e um domínio similar ao sítio ativo da iduronidase, um membro do clan *GH-A-fold* das hidrolases de glicosídeos. Enquanto D1 e D3 são proteínas de meia-vida longa localizadas na membrana plasmática, a D2 é uma proteína residente do retículo endoplasmático com meia-vida de apenas 20min. A inativação da D2 é mediada por conjugação seletiva à ubiquitina, catalisada pela UBC-7, um processo que é acelerado pela catálise do T_4, mantendo assim a homeostase local do T_3. Além disso, a D2 interage e é substrato das VDU1 e VDU2 (pVHL-*interacting deubiquitinating enzymes*), fazendo com que sua desubiquitinação regule o suprimento de hormônio tiroideano ativo em células que expressam D2. **(Arq Bras Endocrinol Metab 2004;48/1:16-24)**

Descritores: Desiodases; Hormônios tireoidianos; Selenocisteína; T_3 homeostase

Recebido em 14/10/03
Aceito em 20/10/03

THE THREE DEIODINASES, enzymes that activate thyroxine (T_4) and inactivate both T_4 and T_3, are present in all vertebrates studied so far, indicating that thyroid hormone deiodination is an intrinsic component of the thyroid hormone homeostasis. In a nutshell, their relevance resides on the fact that T_4 is a long-lived ($t_{1/2}$ is ~7 days in humans) pro-hormone molecule that must be activated by deiodination to the short-lived biologically active T_3 ($t_{1/2}$ is ~1 day) in order to initiate thyroid hormone action. T_3 modulates gene expression in virtually every vertebrate tissue through ligand-dependent transcription factors, the thyroid hormone receptors (TR). The deiodination of T_4 to T_3 occurs in the phenolic (outer or 5')-ring of the T_4 molecule and is catalyzed by two iodothyronine deiodinases, i.e. D1 and D2. As a counter point to the activation pathway, both T_4 and T_3 can be irreversibly inactivated by deiodination of the tyrosyl (inner or 5)-ring, a reaction catalyzed by D3, the third member of the deiodinase group.

In both experimental animals and humans the coordinated changes in the expression and activity of these enzymes ensure thyroid hormone homeostasis and the constancy of T_3 production, constituting a major mechanism for adaptation to changes in the ingestion of iodine, starvation and changes in environmental temperature (reviewed in [1]). The study of animals with natural deficiency of D1 (C3H mouse) or targeted disruption of D2 (Dio2$^{-/-}$) or D3 (D3$^{-/-}$) genes has not only confirmed but revealed new intricacies about the critical role played by these enzymes in thyroid hormone homeostasis (2-5).

Until recently, a frustrating aspect of the deiodinase field was the lack of clinical entities associated with deiodinases dysfunction. This can be explained by the existence of multiple redundant mechanisms involved in T_3 homeostasis. In order to have clinical manifestations of deiodinase dysfunction a patient would have to have alterations in serum T_3 causing hypo- or hyperthyroidism. However, serum T_3 is contributed not only by D1 and D2, but also by direct thyroidal secretion. One could argue that a genetic deficiency of either enzyme would be clinically silent because the other three remaining mechanisms would compensate for the missing pathway. This hypothetical symptoms-free human phenotype is confirmed in C3H (2,6-8) and in the Dio2$^{-/-}$ mice (3,5). However, a preliminary characterization of the Dio3$^{-/-}$ mouse indicates a phenotype of central hypothyroidism, suggesting that the hypothalamic clearance of T_3 is compromised due to the lack of D3 activity (9). In addition, the continued search for such conditions has recently paid off and overexpression or the presence of a deiodinase gene polymorphism is indeed associated with pathological states such as consumptive hypothyroidism in newborns (10) and adults (11), mesothelioma (12), obesity and insulin resistance (13), emphasizing the key role played by the deiodinases in thyroid hormone homeostasis.

A Breakthrough: The 3D Structure of the Deiodinases is Conserved

The three deiodinase proteins (D1, D2 and D3) show considerable similarity (~50% sequence identity). All are integral membrane proteins of 29-33kDa, and have regions of high homology in the area surrounding the active center (14-16). However, because they have such differences in substrate preference, kinetic properties, half-lives and sensitivity to PTU, the general idea was that the three dimensional (3D) structure of these proteins was substantially different, a concept that was kept alive by the inherent difficulties in determining high-resolution structural data for a functional membrane-anchored enzyme.

Insights into the structures of these proteins were obtained through the fortuitous use of protein modeling through hydrophobic cluster analysis (HCA) (17). This is a sensitive method based on the fundamental principles of protein fold and on a two-dimensional transposition of sequences, allowing the resolution of a sequence into its regular secondary structures, centered on the so-defined hydrophobic clusters.

Based on the HCA analysis, it is clear that the three deiodinases share a common general structure composed of a single trans-membrane segment, which is present in the N-termini of D1, D2 and D3, and several clusters, typical of α-helices or β-strands, corresponding to core secondary structures of the deiodinase globular domains (figure 1). A striking common feature is the presence of the thioredoxin (TRX) fold, defined by the βαβ and ββα motifs. It is interesting that, within the canonical TRX fold, the relationship between the βαβ and ββα motifs is locally interrupted by interfering elements. These sequences correspond to distinct secondary structure elements added to the canonical TRX fold core, a feature also observed in other proteins of the TRX fold family (18). A unique aspect of the deiodinases, however, is that one of these highly conserved intervening elements share striking similarities with α-L-iduronidase (IDUA; 47% identity with D1 and D3, 60% with D2), a lysosomal enzyme that cleaves α-linked iduronic acid residues from the nonreducing end of the glycosaminoglycans, heparan sulfate and dermatan sulfate

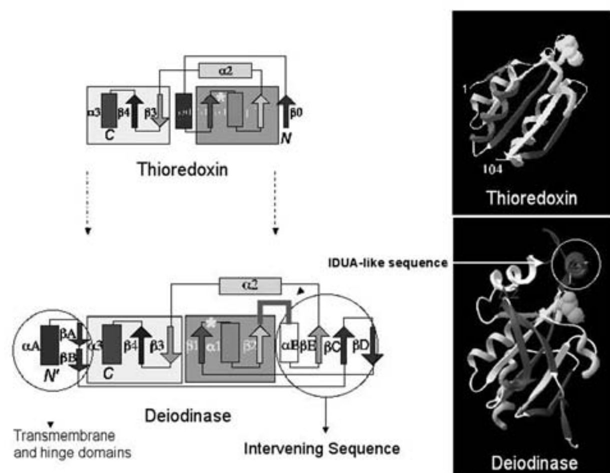

Figure 1. 3D structures and secondary structure organizations of the archetype TRX enzyme and of the overall rough model of the deiodinases deduced from the HCA-based alignment. The transmembrane, hinge and intervening sequences are circled (modified from 17).

(19). Putatively, this local structural mimicry between deiodinases and iduronidase may rely partly on the overall similarity of their substrates, T_4 (or T_3) and sulfated α-L-iduronic acid, respectively, both based on O-linked hexagonal rings substituted by bulky groups ortho to the linker.

The 3D general model of the deiodinases predicts that the active center is a pocket defined by the β1-α1-β2 motifs of the TRX-fold and the IDUA-like insertion (figure 2). The most striking feature of this pocket is the presence of the rare amino acid Selenocysteine (Sec), critical for the deiodination reaction catalyzed by all three deiodinases. This was first identified when the rat D1 cDNA became available, the analysis of which revealed the presence of the Sec encoded by UGA, which is recognized in the vast majority of mRNAs as a STOP codon (20). However, a specific RNA stem-loop immediately downstream of the UGA codon allows for the Sec incorporation in the STOP codon. This structure is termed the Sec Insertion Sequence, or SECIS element, which is present in the three deiodinases and all other selenoproteins (21).

There is sound evidence that all three deiodinases can form homodimers when transiently expressed (22,23). However, it is not clear that dimerization is necessary for function or that it even occurs when the enzymes are expressed at endogenous levels. The latter is supported by the finding of activity in higher molecular weight forms (~65kDa) than would be predicted from their respective deduced amino acid sequences (29kDa) (24,25). However, these higher molecular weight forms could reflect associations with other cellular protein(s) not primarily involved in their catalytic function, but which could, for example, regulate half-life, transport or subcellular localization. More studies are necessary to clarify the functional significance of deiodinase dimerization.

Topology and Subcellular Localization: Physiological Implications

The D1 and D2 monomers are Type 1 integral membrane proteins oriented with a small NH_2-terminal extension in the endoplasmic reticulum (ER) lumen and a single transmembrane domain exiting the ER at about position 40 (26,27). This puts the active center of both D1 and D2 in the cytosol. D3, on the other hand, is a Type 2 integral membrane protein with the opposite orientation of D1 and D2, putting its active center inside the ER lumen (during synthesis) and finally the extracellular space (28) (figure 3).

The subcellular location of mature D1 is the plasma membrane. This has been specifically demonstrated in a number of cell types endogenously or transiently expressing the enzyme (22,29,30). Recently, using confocal laser microscopy of human and mouse cell lines, transiently expressed FLAG-tagged D1 was found in the plasma membrane (28). D1 does not co-localize with the ER resident protein BiP, as does D2 (27). This plasma membrane localization has been confirmed by specific biotinylation of D1 by cell-impermeable agents (28). D2, on the other hand, is an

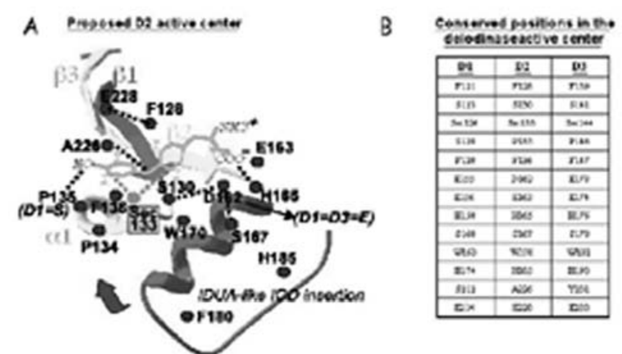

Figure 2. Schematic representation of the putative active site of deiodinases deduced from sequence alignment and from the associated modeling (figure 1). The positions shown are those of D2 and the table contains the corresponding positions and residues in D1 and D3. The IDUA-like IOD insertion likely constitutes a cap that may cover the active site upon ligand binding (modified from 17).

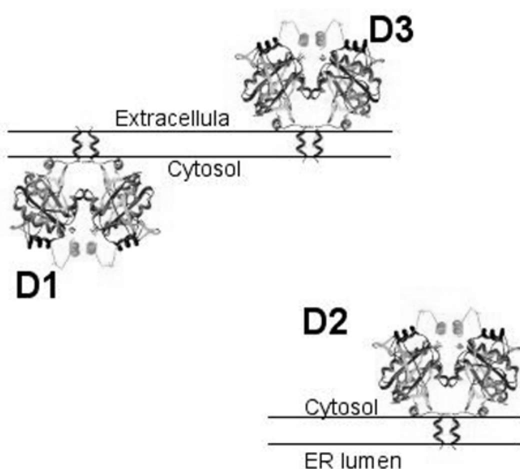

Figure 3. Schematic representation of D1, D2 and D3 dimers with their respective orientations and subcellular localization based on references (26-28).

ER-resident protein. Immunofluorescent confocal microscopy of human and mouse cells transiently expressing FLAG-tagged D2 show that D2 co-localizes with BiP. Endogenously expressed D2 also co-localizes with BiP in a human mesothelioma cell line (MSTO-211H cells) (12).

The subcellular localization of D3 was studied using similar techniques (28). Endogenously and transiently expressed FLAG-tagged D3 was identified in the plasma membrane. It co-localizes with Na, K-ATPase α, with the early endosomal marker EEA-1 and clathrin but not with two ER resident proteins. Most of the D3 molecule is extracellular and its plasma membrane assignment is confirmed by biotinylation with a cell-impermeant probe. There is constant internalization of D3 that is blocked by sucrose or methyl-β-cyclodextrin containing medium. Exposing cells to a weak base such as primaquine increases the pool of internalized D3, suggesting that D3 is recycled between plasma membrane and early endosomes. Such recycling probably accounts for the much longer half-life of D3 (12h) than the thyroxine (T_4) activating members of the selenodeiodinase family, Type 1 (D1; 8h) or Type 2 (D2; 2h) deiodinase.

Thus, a plasma membrane location for D1 could be viewed teleologically as offering ready access of circulating reverse T_3 and T_4 to the enzyme as well facilitating the re-entry of the T_3 produced from T_4 back into the plasma. The localization of D1 in the plasma membrane is in striking contrast to the ER localization of D2 (27). This differential subcellular localization of D1 and D2 may explain why there is such a minimal contribution of the T_3 generated by D1 to the intranuclear T_3 in contrast to large fraction of D2-generated T_3 to this compartment (31-34). The extracellular location of D3 gives ready access to circulating thyroid hormones, explaining its capacity for rapid inactivation of circulating T_4 and T_3 in patients with hemangiomas and its blockade of the access of maternal thyroid hormones to the human fetus (10,35,36).

The Ubiquitination Pathway Inactivates D2

D2 is considered the critical homeostatic T_3-generating deiodinase due to its substantial physiological plasticity. For example, D2 responsiveness to cAMP constitutes the basis for its rapid adrenergic stimulation in BAT, human skeletal muscle and thyroid, linking D2 expression with the sympathetic nervous system and widening the spectrum of environmental and endogenous stimuli that can potentially influence adaptive T_3 production (1 for review).

A number of transcriptional and post-translational mechanisms have evolved to ensure limited expression and tight control of D2 levels, which is inherent to its homeostatic function. The D2 mRNA in higher vertebrates is more than 6kb in length, containing long 5' and 3' untranslated regions (UTRs). The D2 5'UTRs are greater than 600 nucleotides and contain 3–5 short open reading frames (sORFs), which reduce D2 expression by as much as 5-fold (37). Alternative splicing is another mechanism that regulates D2 level, as mRNA transcripts similar in size to the major 6- to 7-kb D2 mRNAs, but not encoding an active enzyme, are present in both human and chicken tissues. D2 levels can also be regulated by AUUUA instability motifs located in the 3'UTR of D2 mRNA as deletion of 3.7-kb from this region increases D2 activity approximately 3.8-fold due to an increase in D2 mRNA half-life (37).

D2 activity/mRNA ratios are variable, indicating that there is significant post-translational regulation of D2 expression (38). In fact, the decisive D2 property that characterizes its homeostatic behavior is a short half-life (~20min) (39) that can be further reduced by exposure to physiological concentrations of its substrate, T_4, and in experimental situations, reverse T_3 or T_3 (39-45). This constitutes a rapid, potent generalized regulatory feedback loop that efficiently controls T_3 production and intracellular T_3 concentration based on how much T_4 is available. The potency of the iodothyronines to induce loss of D2 activity mirrors the enzyme's affinity for the substrate, indicating that enzyme-substrate interaction must occur in order to induce loss of D2 activity.

How is loss of D2 activity mediated? Important metabolic pathways often contain key rate-limiting enzymes whose half-lives can be modified by selective proteolysis. This process is frequently mediated by the ubiquitin (Ub)-proteasome system by which target proteins are marked for destruction by conjugation to Ub, a ~8kDa protein. The ubiquitinated proteins are subsequently recognized and degraded by the proteasomes (46,47). Indeed, ubiquitination and proteasomal degradation are deeply implicated in the post-translational regulation of D2 activity. The first evidence was obtained in GH4C1 cells in which the half-life of the endogenous D2 was noted to be stabilized by MG132, a proteasome inhibitor (48). Substrate-induced loss of D2 activity was also inhibited by MG132 in such cells, indicating that both pathways affecting loss of D2 activity were mediated by the proteasomes. This implies that the loss of D2 activity is, at least partially, due to proteolysis, a premise that was confirmed after the levels of immunoprecipitable labeled D2 were shown to parallel D2 activity, both under basal conditions and after exposure to substrate (49).

Selection of specific proteins for proteolysis is usually achieved at the level of Ub conjugation, a process that involves recognition of amino acid-sequences in the target protein by the ubiquitinating enzymatic machinery. No such sequence(s) was identified for D2 yet. The first step is activation of Ub by ATP, a process catalyzed by the E1 enzyme. The next step, target recognition, is coordinated by the combined actions of a series of Ub-conjugating enzymes (E2s) and Ub-ligases (E3s). There are approximately a dozen E2s or E2-related proteins, which share a conserved catalytic domain of approximately 150 amino acids (50). Individual E2s are involved in different cellular processes and, therefore, in the ubiquitination of different classes of substrate proteins. E3s, on the other hand, are more abundant and with no overt sequence homology, are thought to be largely responsible for the high degree of specificity of protein ubiquitination (47).

Evidence of D2 ubiquitination and E1 involvement was obtained in cells expressing a temperature sensitive E1. At the restrictive high temperature, which inactivates the E1, D2 activity and protein levels are stabilized, even when protein synthesis is inhibited or D2 substrates are present (51). Ub-D2 conjugates are high molecular weight proteins (100-300kDa) easily identified in lysates of cells transiently expressing an epitope tagged D2. In such system, Ub-D2 conjugates behave as expected, i.e. they are increased in cells exposed to D2 substrates such as T_4 and decreased if E1 activity is blocked (51). Exposure to the proteasome uptake inhibitor MG132 also increases Ub-D2 conjugates because proteasome blockade does not interfere with ubiquitination. An important observation is that, under a number of conditions, D2 activity in a cell lysate correlates with the levels of D2 protein and not Ub-D2, indicating that D2 is inactivated by ubiquitination. Interestingly, under the same conditions, D1 and D3 are not ubiquitinated that is in agreement with their relatively long (>12h) half-lives (51).

To identify additional proteins involved in D2 ubiquitination, a Cysteine mutant of D2 was expressed in the yeast *S. cerevisiae*, a cell model in which the Ub-proteasome system is well characterized and D2 displays its typical cellular and molecular properties. Especially important, D2 was highly ubiquitinated and maintained its short half-life and sensitivity to substrate exposure, and its degradation was blocked by inhibitors of the proteasome uptake. Interestingly, D2 was stabilized in yeast strains that lack specific E2s, Ubc6p or Ubc7p. Both of these E2s are involved in the ER-associated degradation (ERAD) process, in agreement with the fact that D2 is an ER-resident protein. Importantly, in the yeast that lacked the E2 proteins the substrate-induced loss of D2 activity and proteolysis were also impaired. On the other hand, no difference in D2 levels was detected in a yeast strain deficient in Ubc1p; an E2 involved in the degradation of unfolded protein, thus confirming that D2 ubiquitination in the yeast system is specific (52).

The recent identification of murine homologues of UBC6 and UBC7, MmUBC6 and MmUBC7 (53), has made it feasible to investigate the relevance of UBC6 and UBC7 for D2 ERAD in mammalian cells. The major novel finding is that there is a high affinity, specific physical association between MmUBC7 and a carboxy region (aa 169-234) of the D2 enzyme (54), a region that is exposed to the cytosol according to prior topological analysis (27). While direct binding of E2s has been proposed for certain substrates (47,55-57), current models of ubiquitination suggest that E2s play only a secondary role in substrate recognition, with E3 enzymes being the major specificity determinant (47). It is therefore likely that the D2-MmUBC7 association is mediated by an as yet unknown E3 or other adaptor protein(s) present in the reticulocyte lysate.

To understand the individual role of each of these E2s in D2 ubiquitination, we pursued a previously described functional strategy in which the ERAD mechanism is saturated via overexpression of inactive UBC6 and/or UBC7 mutants (53). In this setting D2 activity was stabilized to a degree that can be explained

by changes in D2 protein half-life (54). That both UBC6 and UBC7 must be neutralized in order to achieve D2 stabilization suggests that redundancy exists at the level of the E2, i.e. either UBC6 or UBC7 can catalyze D2 ubiquitination. Thus, co-expression of the individual E2 mutants had no effect on D2 ERAD. However, the fact that MmUBC6 does not bind with the same affinity to the D2 ERAD complex, as does MmUBC7 suggests that there may be a preference for UBC7-mediated D2 ERAD (54) (figure 4). In fact, it has been suggested that UBC7 rather than UBC6 is dominant in ERAD (53,58,59), and controversy exists as to the role of UBC6 in the ubiquitination of some ERAD substrates including the T-cell receptor subunits α and CD3-δ (60).

Reversible Ubiquitination Rescues D2 From Irreversible Proteolysis

Fusion of the 8-amino acid FLAG sequence to the COOH but not the NH_2 terminus of D2 prolongs its activity half-life and increases the size of the Ub-D2 pool 20- to 30 fold (51), suggesting that D2 ubiquitination is reversible, as not all Ub-D2 undergoes proteolysis. Enzymatic de-ubiquitination of Ub-D2 occurs *in vitro* (61) and could explain recycling *in vivo*. Using the COOH-terminal portion of D2 in a yeast-two-hybrid screening we recently identified D2 as the only known specific substrate of the Von Hippel Lindau protein (pVHL)-interacting deubiquitinating enzyme (VDU1) and VDU2 (62), which in turn are the first ubiquitin-specific processing proteases (UBP) known to specifically deubiquitinate an

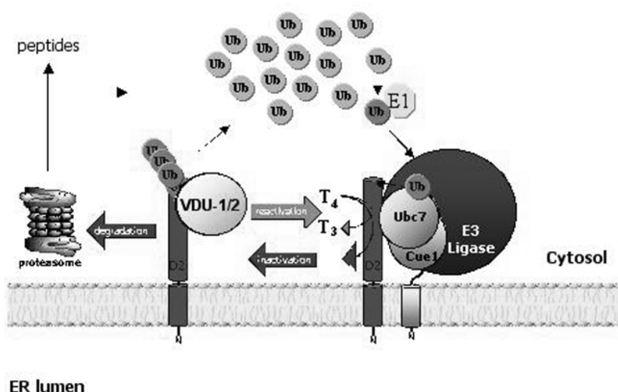

Figure 4. Schematic representation of D2 ubiquitination, deubiquitination and proteasomal degradation. Ub is ubiquitin; E1 is the enzyme that activates Ub; Ubc7 is the Ub conjugase; VDU1/2 catalyze D2 deubiquitination; Cue1 is a ER-docking protein for Ubc7 (modified from ref. 76).

ERAD substrate. These results show that protein recognition is not only involved 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 (63,64). Although hundreds of UBP enzymes have been cloned, only a few examples of substrate recognition by UBP enzymes have been reported and, to our knowledge, none are ER-resident proteins (65-69). Confocal studies indicate that both VDUs co-localize with D2, itself an integral membrane ER-resident protein. Although present in the particulate fraction and not in cytosol it is not clear based on their hydrophobic profile whether VDU1/2 are integral membrane proteins (62). Their physical colocalization with D2, however, provides the opportunity for catalysis and D2 deubiquitination.

VDU1 catalyzed D2 deubiquitination is an important part of the adaptive mechanism that regulates thyroid hormone action (figure 4). In stimulated brown adipocytes normal D2 induction increases intracellular T_3 production, resulting in isolated tissue thyrotoxicosis (5,34,70). 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 (5). Our results demonstrate that increased VDU1-catalyzed deubiquitination of a pool of Ub-D2 and its rescued from proteasomal degradation is an integral part of this mechanism. VDU1 mRNA levels are markedly up-regulated by cold exposure or NE, amplifying the transcriptional increase in D2 activity and hence T_3 production by approximately 2.5 fold. Even though UBP induction is known to play a physiological role in a number of cellular processes (71-75), 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. The finding that VDU1 and VDU2 are co-expressed with D2 in many human tissues, including brain, heart and skeletal muscle (1,63), indicates that the importance of this mechanism may extend well beyond thermal homeostasis to include brain development, cardiac performance, glucose utilization and energy expenditure.

ACKNOWLEDGEMENT

Supported by NIH grant DK58538.

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