Selective Proteolysis of Human Type 2 Deiodinase: A Novel Ubiquitin-Proteasomal Mediated Mechanism for Regulation of Hormone Activation

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We investigated the mechanism by which T_4 regulates its activation to T_3 by the type 2 iodothyronine deiodinase (D2). D2 is a short- lived ($t_{1/2}$ 50 min), 31-kDa endoplasmic reticulum (ER) integral membrane selenoenzyme that generates intracellular T_3. Inhibition of the ubiquitin (Ub) activating enzyme, E1, or MG132, a proteasome blocker, inhibits both the basal and substrate-induced acceleration of D2 degradation. Using a catalytically active transiently expressed FLAG-tagged-NH_2-D2, we found rapid synthesis of high molecular mass (100–300 kDa) Ub-D2 conjugates that are catalytically inactive. Ub-D2 increases when cells are exposed to D2 substrate or MG132 and disappears rapidly after E1 inactivation. Fusion of FLAG epitope to the COOH terminus of D2 prolongs its half-life approximately 2.5-fold and increases the levels of active and, especially, Ub-D2. This indicates that COOH-terminal modification interferes with proteasomal uptake of Ub-D2 that can then be deubiquitinated. Interestingly, the type 1 deiodinase, a related selenoenzyme that also converts T_4 to T_3 but with a half-life of >12 h, is inactivated but not ubiquitinated or degraded after exposure to substrate. Thus, ubiquitination of the ER-resident enzyme D2 constitutes a specific posttranslational mechanism for T_4 regulation of its own activation in the central nervous system and pituitary tissues in which D2-catalyzed T_4 to T_3 conversion is the major source of intracellular T_3. (Molecular Endocrinology 14: 1697–1708, 2000)

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INTRODUCTION

Various intracellular regulatory pathways can be modified by selective proteolysis of key rate-limiting enzymes. This process is frequently mediated by the proteasome system in which different metabolic signals stimulate uptake and proteolysis by the proteasomes. Well known examples include cytosolic enzymes such as ornithine decarboxylase (1) and endoplasmic reticulum (ER) transmembrane protein hydroxy-methylglutaryl-coenzyme A reductase (2). In many cases selective proteolysis is preceded by conjugation to ubiquitin (Ub), a step that is activated by exposure of cells to specific substrates. In *Saccharomyces cerevisiae*, for example, glucose-containing medium induces the metabolic transition from gluconeogenesis to glycolysis by ubiquitination and degradation of fructose-1,6-biphosphatase, a key regulatory enzyme of gluconeogenesis that catalyzes the hydrolysis of fructose-1,6-bisphosphate to generate fructose-6-phosphate and inorganic phosphate (3). This degradation process has been termed "catabolite inactivation" and operates for other key enzymes in this pathway as well, including cytosolic malic enzyme, phosphoenolpyruvate kinase, and isocitrate lyase (4). The specific mechanism(s) by which catabolites induce the proteolytic cascade is poorly understood.

In vertebrate cells, basal and substrate-induced selective enzyme proteolysis is also involved in the control of transformation of T_4 to the active hormone T_3 , the first step in thyroid hormone action. Two enzymes, the types 1 (D1) and 2 (D2) iodothyronine deiodinases, can catalyze this reaction. Along with type 3 deiodinase (D3) that inactivates T_4 and T_3, these integral membrane selenoproteins constitute a homeostatic system that controls the intracellular concentration of active thyroid hormone within human tissues. Whereas

D1, expressed primarily in liver and kidney, is considered to be the major source of circulating T_3, D2 catalyzes the local production of T_3 in the central nervous system, pituitary gland, and brown adipose tissue (5). As a result, thyroid hormone receptor (TR) occupancy by T_3 is higher in these tissues than is the case in cells in which plasma is the only source of intracellular T_3. In addition, the effects of a decrease in plasma T_4, such as occurs in iodine deficiency, are mitigated by a rapid compensatory increase in D2 activity (5). One key feature of D2 that allows such plasticity is its short half-life (<1 h) (6).

Considerable evidence indicates that D2 regulation by its substrates is posttranslational (7–9) and recent data implicate the proteasome system (10, 11). Indeed, in pituitary tumor cells, proteasomal inhibitors (MG132 or lactacystin) stabilize D2 activity for several hours in the presence of cycloheximide (CX) or the D2 substrates T_4 or rT_3 (10). Parallel reductions of transiently expressed ^{75}Se-labeled D2 and D2 activity occur after exposure of cells to CX and/or rT_3, indicating that this reduction is due to catabolism of the protein rather than to an alteration in its structure (11). Single amino acid changes in the active center of D2, which either raise the Michaelis-Menten constant (K_m) for substrate approximately 1,000-fold or block its catalytic activity, impair or eliminate substrate-induced loss of D2 (11). These results suggest that interaction of substrate with D2 selectively targets the protein for degradation by the proteasome system. Despite this, an ubiquitinated D2 intermediate has not been identified nor has the role of catalysis in its production been clarified.

Proteins containing selenocysteine (Sec) are synthesized slowly due to the complex mechanisms required to suppress the stop codon function of the Sec codon, UGA (12). This and its short half-life result in low cellular D2 concentrations. D2 protein contains few immunogenic peptide sequences, resulting in difficulties in the generation of high-affinity antibodies suitable for Western blots (11). Accordingly, to enhance the possibility of detecting ubiquitinated D2, we employed a mutated D2 enzyme in which Cys is substituted for Sec 133 in the active center. This protein is transiently expressed at levels approximately 100-fold higher than the native D2 but is catalytically active and subject to the same substrate regulatory pathways as is the native enzyme (11). We also labeled D2 with FLAG, an epitope for which there is a highly specific antibody. By transiently expressing catalytically active FLAG-tagged D2, we have identified ubiquitinated-D2 and show that its levels are increased by exposure of cells to substrate. We also found that the Ub-activating enzyme, E1, is required for Ub conjugation to D2 and for the substrate-induced acceleration of that process. Surprisingly, while we have confirmed previous results showing that substrate also reduces D1 activity, this T_4 activating enzyme is not ubiquitinated. This indicates that ubiquitination of D2 is a specific mechanism that confers rapid posttranslational regulation by T_4 of its activation to T_3. This is a unique example of substrate-induced selective proteolysis that involves ubiquitination of an ER resident protein. To our knowledge, it is the first demonstration that such a regulatory pathway controls activation of a hormone.

RESULTS

An Active E1 Ub-Activating Enzyme Is Required for D2 Proteolysis

In the first group of experiments, wild-type D2 protein (D2) was transiently expressed in a Chinese hamster cell line (CHO-ts20), which has a temperature-sensitive E1, the Ub-activating enzyme. These cells retain a functional ubiquitination pathway when cultured under the permissive temperature (<35 C). At 40 C, the heat-sensitive E1 has less than 10% of its normal activity (13). Ts20 cells grown at 30 C were transfected in pairs with D2-expressing plasmid. This technique leads to transient expression of equal quantities of protein as assessed by measuring GH in the media 24 h later (see *Materials and Methods*). After 48 h, one plate of each pair was placed at 40 C while the other remained at 30 C. At the indicated times, cells were processed for D2 activity (Fig. 1A). In three independent experiments the D2 activity increased an average of approximately 30% at the restrictive temperature (Fig. 1B), whereas no changes were detected in similarly treated wild-type CHO cells (data not shown).

We next determined whether inactivation of E1 would affect the half-life of D2 or the effects of substrate to reduce its activity. rT_3 was chosen as D2 substrate because it is metabolically inactive, its deiodination product is also inactive, and the D2 $K_m(rT_3)$ is similar to that of T_4. In fact, in a previous publication (11) we showed that both T_4 and rT_3 cause similar acceleration of D2 inactivation. Plates containing ts20 cells transiently expressing D2 were kept at 30 C or placed at 40 C for 4 h. Cycloheximide (100 μM) or rT_3 (30 nM) was added to one plate of each pair (Fig. 1C). These treatments caused an approximately 20% reduction in D2 activity in cells at 30 C ($P < 0.05$). However, at 40 C, these agents had no significant effect on D2 activity ($P > 0.05$ *vs.* time 0; $P < 0.05$ *vs.* 30 C) (Fig. 1, D–E). The effects of CH or rT_3 in wild-type CHO cells transiently expressing D2 were not affected by incubation at 40 C (data not shown). These results indicate that E1 is rate limiting in the basal and substrate-induced changes in D2 activity.

To confirm that there was a correlation between enzyme activity and D2 protein we evaluated changes of ^{75}Se-labeled D2 under the same conditions. Paired plates of ts20 cells transiently expressing wild D2 were labeled with $Na_2[^{75}Se]O_3$, and 24 h later one plate was shifted to 40 C for 4 h (Fig. 2A). The plate lysates were processed for immunoprecipitation (IP) using D2 antiserum and the precipitates analyzed by SDS-PAGE. The 31-kDa ^{75}Se-D2 band increased 1.8 $\pm$ 0.6 fold ($P < 0.05$) in cells at 40 C *vs.* those at 30 C, again consistent with im-

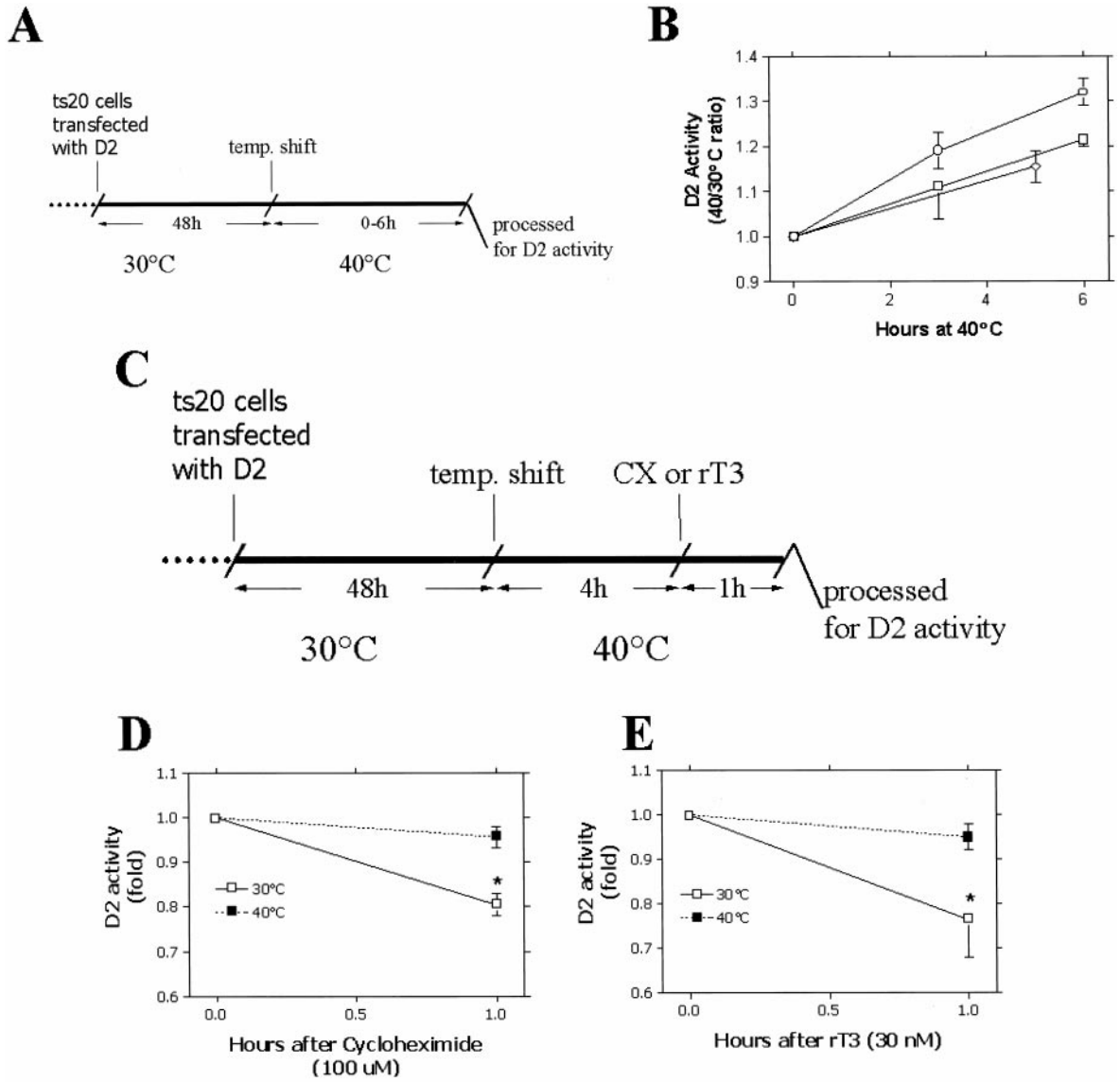

Fig. 1. Inhibition of the Ub-Activating Enzyme, E1, Blocks D2 Degradation

A, Paired plates of ts20 expressing wild-type hD2 were kept at the permissive temperature (30 C) for 48 h. B, At that time one plate of each pair was transferred to the restrictive temperature (40 C), and D2 activity was assayed at the indicated times and expressed relative to D2 in cells kept at 30 C. The data shown are the mean ± SD (n = 2) of three different experiments. C, Similar protocol to evaluate effects of E1 inhibition on D2 degradation or substrate-induced proteolysis. After 4 h, 100 μM CX (D) or 30 nM rT_3 (E) was added and D2 measured 1 h later and shown relative to control at 30 C. *, $P < 0.05$ *vs*. 0 time-point. There were no significant differences between the 4 and 5 h time points for cells kept at the restrictive temperature (40 C) in experiments D and E.

paired ubiquitination (and subsequent proteolysis) of D2 at the restrictive temperature (Fig. 2, B and C) as the explanation for the increased D2 activity. However, no ^{75}Se-labeled higher molecular mass proteins were visualized in the control samples as would be expected if ubiquitination of D2 was occurring under these circumstances (Fig. 2B).

Identification of Ub-D2

To enhance the possibility of detecting Ub-D2, HEK-293 cells transiently expressing FLAG-NH_2-cysD2 protein (Fig. 3A) were treated with 100 μM CX or 30 μM rT_3, and sonicates were processed for Western blotting with anti-FLAG antibody (Fig. 4A). The ~32 kDa FLAG-NH_2-cysD2 band behaved similarly to the ~31 kDa ^{75}Se-D2 band detected in the IP of HEK-293 cells reported previously (11). It disappeared with a half-life of approximately 2 h in the presence of CX and was also decreased by treatment with rT_3. Both effects were blocked by concomitant exposure to 10 μM MG132, an inhibitor of proteasomal proteolysis (Fig. 4B). D2 activity paralleled the level of FLAG-NH_2-

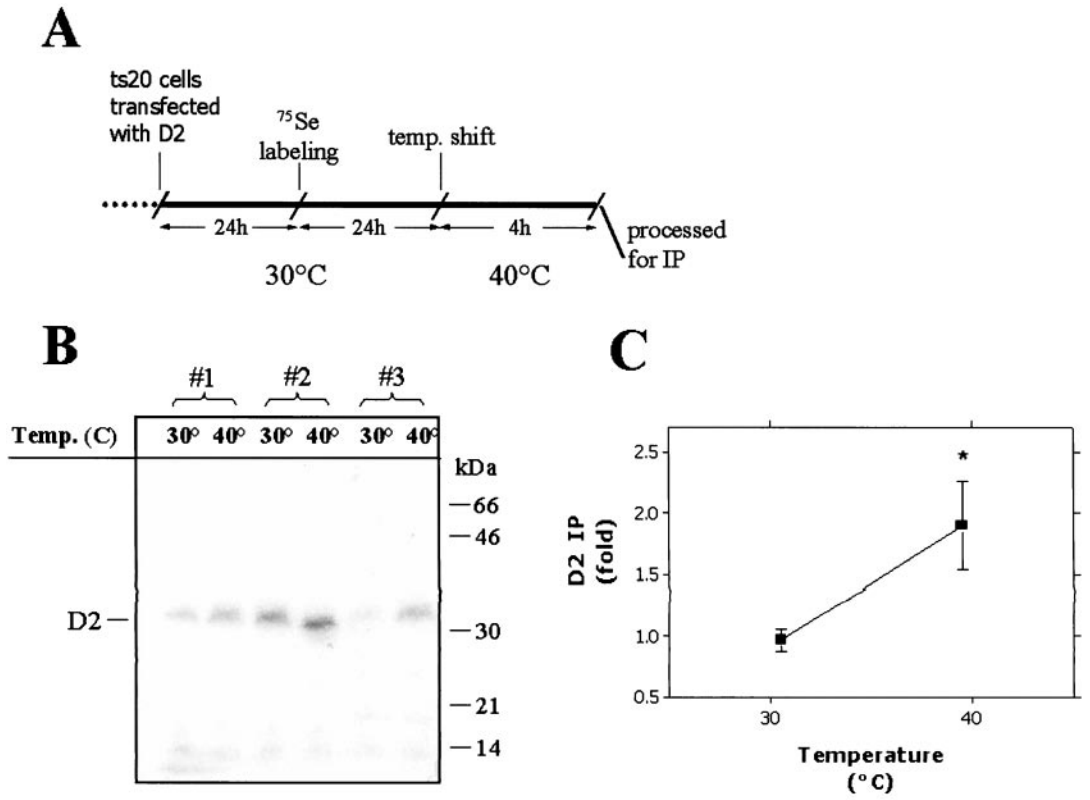

Fig. 2. IP of hD2 Transiently Expressed in 75Se-Labeled ts20 Cells

A,. Ts20 cells transfected in pairs expressing wild-type hD2 were labeled with ^{75}Se. After 1 day, one plate of each pair was transferred to 40 C. After 4 h the D2 was IP'd with an anti-D2 antiserum. B, IP pellets were resolved by 12% SDS-PAGE in three separate experiments, nos. 1, 2, and 3. C, Autoradiograph densitometric analysis of the gel in Fig. 2B, shown relative to control at 30 C. *, $P < 0.05$ *vs*. cells kept at 30 C.

cysD2 protein, as visualized by the Western blot (Fig. 4, B and C). Most importantly, five high molecular mass bands (100–300 kDa) were observed in the Western blot, consistent with the formation of polyubiquitinated D2 (Fig. 4D). These bands constituted 10–20% of the immunoreactive FLAG protein in the cells.

To confirm that these bands were indeed ubiquitinated FLAG-D2, sonicates of HEK-293 cells transiently expressing FLAG-D2 were affinity purified on an anti-FLAG agarose matrix. The samples were then stained with an anti-Ub antiserum (Fig. 4E). Three 110- to 300-kDa bands, similar in size to those seen with the anti-FLAG antibody in Fig. 4D, reacted with the anti-Ub antiserum. Stripping the anti-Ub antibodies from the blot and reprobing it with anti-FLAG antibody confirmed that these proteins were ubiquitinated FLAG-cysD2 (Fig. 4E). Microsomal and cytosolic fractions were prepared from sonicates of HEK-293 cells, and FLAG-cysD2 as well as Ub-FLAG-cysD2 were found to be localized predominantly in the particulate fraction (Fig. 4F).

If the high molecular mass FLAG-D2 proteins are ubiquitinated, then it should be possible to block their formation by inactivating E1 in the ts20 cells. Increasing the incubation temperature to 40 C for 2 h markedly reduced the amount of the high molecular mass FLAG-NH_2-cysD2 complexes in ts20 cells (Fig. 5A). There was also an associated approximately 20% increase in ~32 kDa FLAG protein and D2 activity (100 ± 13 *vs.* $126 \pm 18\%$; $P < 0.05$) in the same cells.

We next investigated whether exposure to substrate would increase the conjugation of Ub to D2. Exposure of HEK-293 cells transiently expressing FLAG-NH_2-cysD2 to 30 μM rT_3 for 2 h caused a 2- to 3-fold increase in the Ub-D2 conjugate bands (Fig. 5B), establishing that substrate-enzyme interaction accelerates the rate of D2 ubiquitination. As expected, the FLAG-NH_2-cysD2 band decreased by 40–50% as did D2 activity (100 ± 20 *vs.* $45 \pm 12\%$; $P < 0.05$). In a complementary experiment, addition of 10 μM MG132 not only increased the ~32 kDa FLAG-NH_2-cysD2 band by 50–60% but also increased the high molecular mass FLAG-NH_2-cysD2 bands, as would be predicted if MG132 blocked their proteasomal uptake (Fig. 5C). The D2 activity of these cells also increased parallel to the FLAG-NH_2-cysD2 bands (100 ± 17 *vs.* $155 \pm 25\%$; $P < 0.05$).

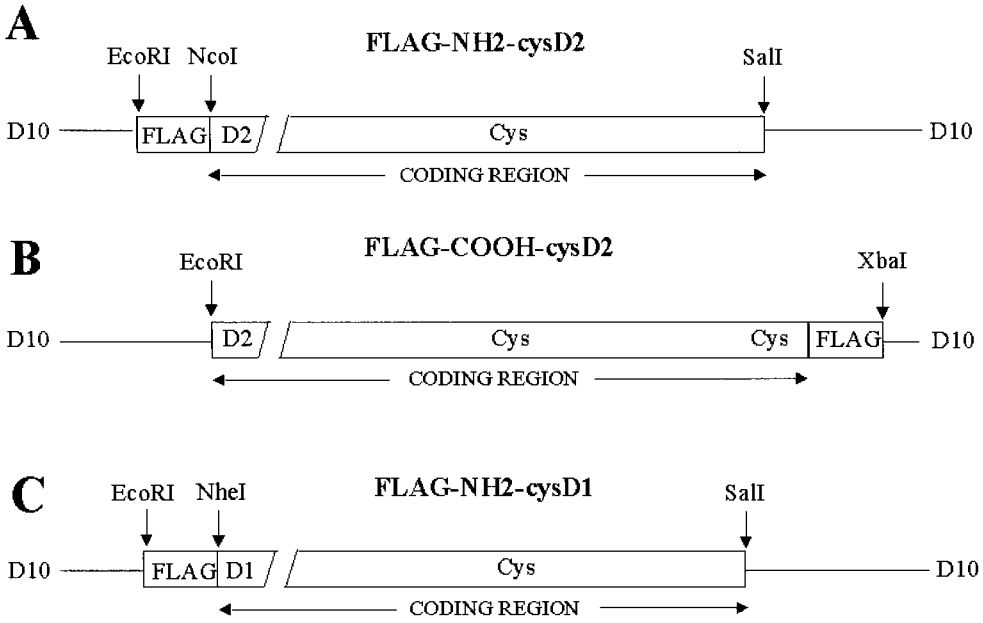

Fig. 3. Schematic Diagram of FLAG-Tagged Deiodinases Containing Sec-to-Cys Mutations in the Active Center.

The COOH Terminus Is Critical for the Proteolysis of Ub-D2

According to the current topological model for the deiodinases, the NH_2 terminus of D2 is located in the lumen of the ER with its catalytic portion in the cytosol. As mentioned previously, fusion of the FLAG sequence to the NH_2-terminus of D2 does not change its degradation rate (Fig. 4B). Surprisingly, the levels of D2 activity (Fig. 6A) and protein (Fig. 6B) were 4-fold higher in cells transiently expressing cysD2 with the FLAG epitope placed at the COOH terminus (Fig. 3B). The amount of Ub-FLAG-COOH-cysD2 was massively increased, about 20- to 30-fold, suggesting that the degradation of Ub-D2 was blocked by the presence of the COOH-terminal FLAG epitope (Fig. 6B). Studies with CX confirmed that the half-life of the FLAG-COOH-cysD2 protein was longer than that of the wild-type D2 or the FLAG-NH_2-cysD2 (Fig. 6, C and D). While CX exposure caused an approximately 50% decrease in the FLAG-NH_2-cysD2 protein over 2 h, FLAG-COOH-cysD2 protein disappeared much more slowly, with a predicted half-life of 4–5 h. Parallel effects occurred in D2 activity.

The longer half-life of the COOH-terminal modified protein could be explained by an impairment of D2 ubiquitination or interference with the further processing of Ub-D2 in the proteasomes. It appears that the latter is the case since the ratio of Ub-FLAG-COOH-cysD2 to unconjugated D2 is several fold higher than that of the FLAG-NH_2-cysD2. It is also notable that additional bands of intermediary molecular masses (40–100 kDa) can be visualized when the film is overexposed. Given the fact that these bands are not present in the negative controls and that they are more abundant in the sonicates from cells transfected with FLAG-COOH-cysD2 than with FLAG- NH_2-cysD2, it is likely that they are lower molecular mass Ub-cysD2 conjugates.

Substrate Causes D1 Inactivation but Not Ubiquitination

As mentioned, the selenoenzyme D1 also catalyzes T_4 to T_3 conversion although the $K_m(T_4)$ for this enzyme ($\sim$2 μM) is approximately 1000-fold higher than is that for D2 (1–2 nM) (14). While D1 has sequence and, presumably, structural similarities to D2, it differs from the latter in having a relatively long half-life ($>$12 h; Fig. 7A). Accordingly, we prepared a FLAG-NH_2-cysD1 protein (Fig. 3C) to allow comparative studies of the degradation pathways of D1 and D2. The FLAG-NH_2-cysD1 was again catalytically active, and the half-life of D1 activity of transiently expressed FLAG-cysD1 was $>$12 h, 6 times longer than that of FLAG-cysD2 (Fig. 7A). Despite much higher transient expression of D1, there was no detectable Ub-FLAG-NH_2-cysD1 conjugate in the HEK-293 cells (Fig. 7B). However, exposure to rT_3 for 24 h caused a 70–80% reduction in D1 activity but no change in the level of FLAG NH_2-cysD1 protein (Fig. 7, C and D). These results indicate that, despite the fact that substrate causes decreases in both D2 and D1 activities, the mechanisms by which those changes occur are quite distinct.

DISCUSSION

The signal(s) that target D2 for ubiquitination are poorly understood. No clear destabilizing sequences

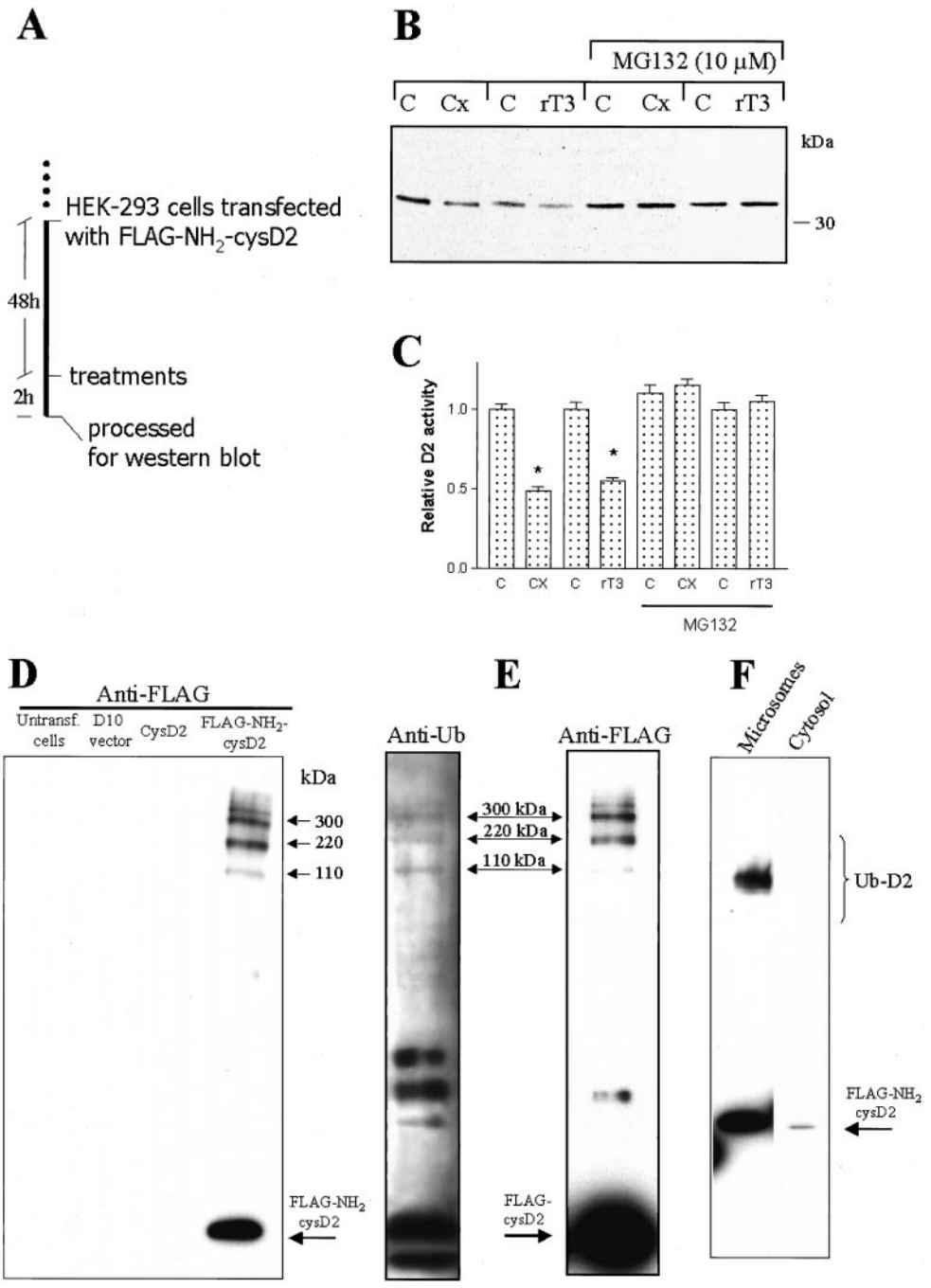

Fig. 4. Western Blot of FLAG-NH_2-cysD2 Fusion Protein Transiently Expressed in HEK-293 Cells

A, HEK-293 cells transfected in pairs with FLAG-NH_2-cysD2-expressing plasmid were treated for 2 h with 100 μM CX, 30 μM rT_3, and/or 10 μM MG132 as indicated. Controls (C) were treated with vehicle (NaOH or DMSO). After 2 h, lysates were resolved in a 12% SDS-PAGE or assayed for D2. B, Western blot using anti-FLAG antibody and C. D2 activity in the same cells. D, Western blot of a second experiment in which HEK-293 cells were prepared as in panel A and processed for the blot. Cell sonicates were resolved by 7.5% SDS-PAGE. The FLAG-NH_2-cysD2 is the 33-kDa protein. Negative controls are indicated and included untransfected cells, cells transfected with empty vector, or cysD2 without FLAG. E, Transiently expressed FLAG-NH_2-cysD2 was isolated using anti-FLAG agarose matrix. After extensive washing the beads were mixed with loading buffer and resolved in a 7.5% SDS-PAGE. Western blot using anti-Ub antibody and Western blot using anti-FLAG antibody after removal of the anti-Ub. The unmarked bands are nonspecific and partially due to reactivity of the peroxidase-labeled second antibody with the anti-FLAG IgG from the matrix. F, Western blot of particulate fraction or cytosol of HEK-293 cells transiently expressing FLAG-NH_2-cysD2. Cell sonicates were centrifuged at 2,500 $\times$ *g* for 10 min and the supernatant was spun at 100,000 $\times$ *g* for 1 h to separate microsomes from cytosol. *, $P < 0.05$ *vs.* control cells.

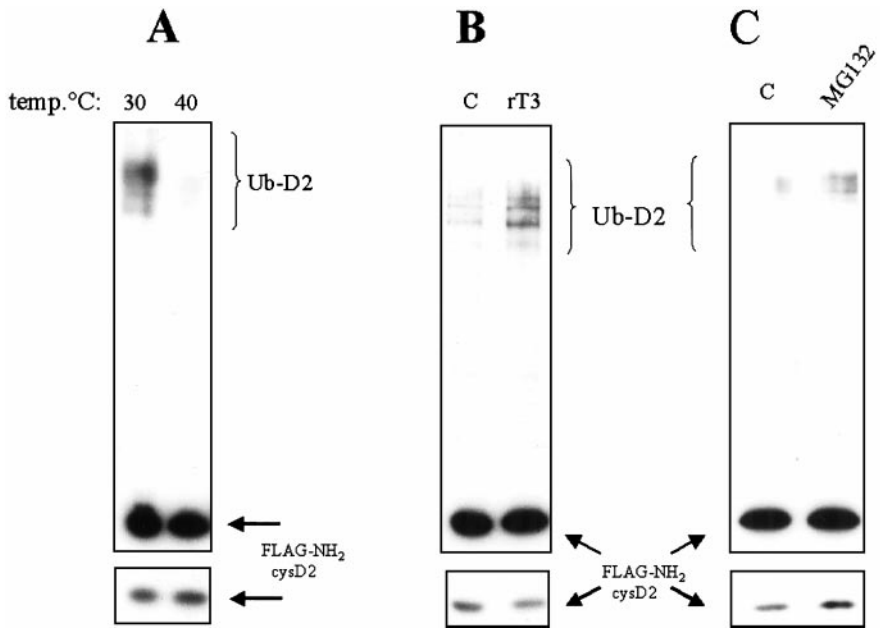

Fig. 5. Inhibition of E1 Blocks the Formation of Ub-D2 Conjugates

A, One of each pair of ts20 cells transiently expressing FLAG-NH_2-cysD2 was transferred from 30 C to 40 C for 2 h. Cell sonicates were processed for Western analysis using anti-FLAG antibody after 7.5% SDS-PAGE. B and C, HEK-293 cells transiently expressing FLAG-NH_2-cysD2 were treated with 30 μM rT_3 (B) or 10 μM MG132 (C) for 2 h. Cell sonicates were processed for Western analysis with anti-FLAG antibody after 7.5% SDS-PAGE of controls (C) and treated cells. *Below* each gel there is a detail of the FLAG-NH_2-cysD2 band in a film properly exposed for that band.

such as PEST elements, N-end rule, or destruction box (15) are present in the D2 molecule even though its half-life is relatively short. This is especially notable since D2 is an integral membrane protein. Our preliminary topological analysis suggests that D2 is an ER integral membrane protein with its NH_2 terminus within the ER (data not shown). Recent studies suggest that both intralumenal and resident ER transmembrane proteins are predominantly degraded by the cytosolic proteasome system after they are dislocated to the cytosol. This includes not only improperly folded proteins, which are subjected to rapid proteasomal degradation, but also resident (properly folded) ER proteins (Ref. 16 for review). One well known example of the latter is the yeast Sec62 protein (17). This protein spans the ER membrane two times and has both termini facing the cytosol. It is a major component of the heptameric Sec complex, a protein-conducting channel of the ER membrane. Like D2, Sec62 is heavily ubiquitinated and degraded by proteasomes. Membrane extraction and proteolysis of Sec62 are coupled mechanisms initiated through the protein's amino terminus and mediated by the proteasomes (17).

Two results suggest that ubiquitination and proteasomal degradation of D2 initiate at the COOH terminus. First we can identify Ub-D2 in particulate fraction of cell sonicates (Fig. 4F). The accumulation of large amounts of Ub-D2 associated with the ER (20–50% of total FLAG protein) indicates that the rate of D2 ubiquitination exceeds that of Ub-D2 proteolysis. This agrees with the hypothesis that proteolysis of ER proteins requires extraction of Ub-conjugated protein from the ER (16). The alternative possibility that the COOH-terminal portion of Ub-D2 is clipped off by an ER-bound proteasome, or by regulated intramembrane proteolysis (18), is unlikely because small FLAG-containing protein fragments ($<$32 kDa) were not detected (Fig. 6). Second, a comparison of the fate of NH_2- and COOH-FLAG-tagged D2 proteins allowed us to gain further insight into the mechanistic aspects of D2 proteolysis. On one hand, conjugation of FLAG to the NH_2 terminus does not alter the half-life of cysD2 (Fig. 4) (11). In contrast, the fusion of the FLAG sequence to the COOH terminus of D2 not only prolonged its half-life but also increased the size of the Ub-D2 pool 20- to 30-fold (Fig. 6). Because both D2 activity and the $\sim$32 kDa protein were increased 3- to 4-fold in cells transfected with FLAG-COOH-cysD2, the increase in the Ub-D2 pool is not due to increased ubiquitination. Rather, it is probably caused by impaired proteasomal extraction/proteolysis of Ub-D2. The accumulated Ub-D2 can then be recycled through Ub isopeptidases to D2. Consequently, D2 half-life, protein levels, and activity are all increased. This is supported by the data in Fig. 5. Likewise, moving ts20 cells to the restrictive temperature decreases Ub-D2 conjugates while it increases D2 protein and activity (Fig. 5). The dynamic equilibrium between Ub-D2 and D2 also explains why D2 activity is stabilized for several hours by exposing CX-treated pituitary tumor cells (10) or HEK-293 cells transiently expressing D2 (11) to MG132. The data presented in Fig. 5 also indicate that

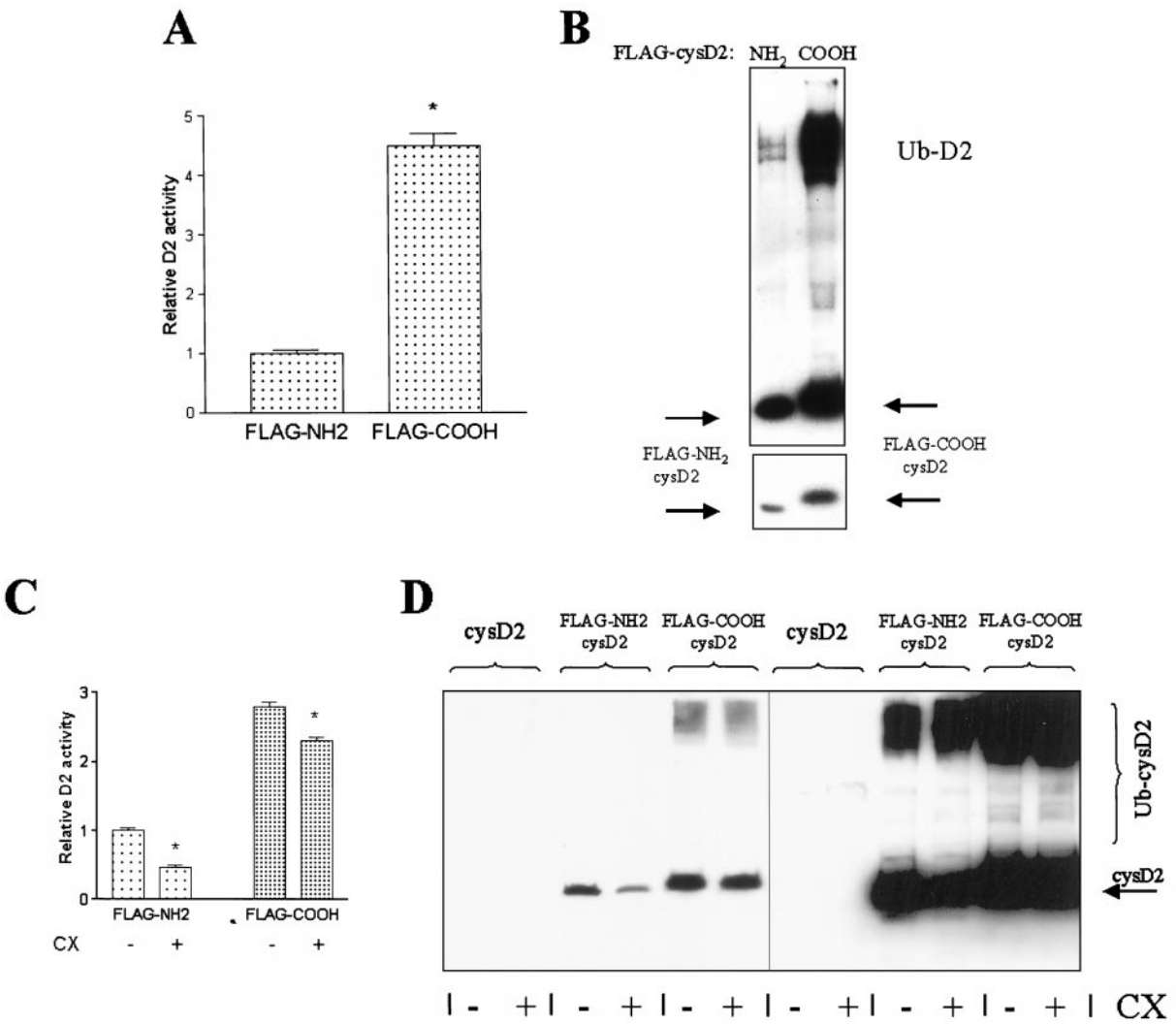

Fig. 6. The COOH-Terminus FLAG Inhibits Proteasomal Uptake of Ub-D2

A, D2 activity quantified from HEK-293 cells transiently expressing either FLAG-NH_2-cysD2 or FLAG-COOH-cysD2. Medium GH used as internal control showed equal transfection efficiency for cells transfected with the FLAG constructs. B, Western blot of the same sonicates with anti-FLAG antibody after 7.5% SDS-PAGE. Below the gel is shown a detail of a shorter exposure. Because of the construct strategy the FLAG-COOH-cysD2 protein is approximately 1 kDa larger than the FLAG-NH_2-cysD2 protein (see *Materials and Methods*). C, HEK-293 cells transiently expressing either FLAG-NH_2-cysD2 or FLAG-COOH-cysD2 were treated with vehicle or CX for 2 h and D2 activity was measured. D**,** A Western blot of the same lysate was probed with anti-FLAG antibody. The two images are from the same immunoblot exposed for different time periods. CysD2 was used as a negative control. The Ub-D2 conjugate bands $<$100 kDa in size are only detected in the film exposed for longer time. *, $P < 0.05$ *vs*. vehicle-treated cells.

D2 activity parallels the levels of D2 protein and not Ub-D2 conjugates, which, therefore, must be catalytically inactive. This indicates that the D2 and Ub-D2 pools are in dynamic equilibrium that shifts toward D2 in the presence of MG132 or toward the formation of Ub-D2 when cells are exposed to substrate. The immediate implication of these findings is that proteasomal uptake can be a limiting step in D2 proteolysis, as has been suggested for Sec62 (17).

Exposure to the substrate rT_3 decreases D2 protein and activity by approximately 50%. The present results reveal that this is due to an increase in D2 ubiquitination since both this and the loss of D2 activity are blocked by E1 inactivation (Figs. 2 and 3). Because D2-substrate interaction is required to increase D2 proteolysis (11), it is possible that postcatalytic structural changes in the D2 protein accelerate the ubiquitination cascade. Alternatively, the redox state of the molecule might play a role. Reducing agents, *e.g*. dithiothreitol, act as cofactors for D2 catalysis *in vitro* by reducing the Se in the enzyme's active center after it is oxidized during the monodeiodination process (19). Oxidation of the Se^- in the native enzyme or SH in the Cys mutant could be the primary signal that acceler-

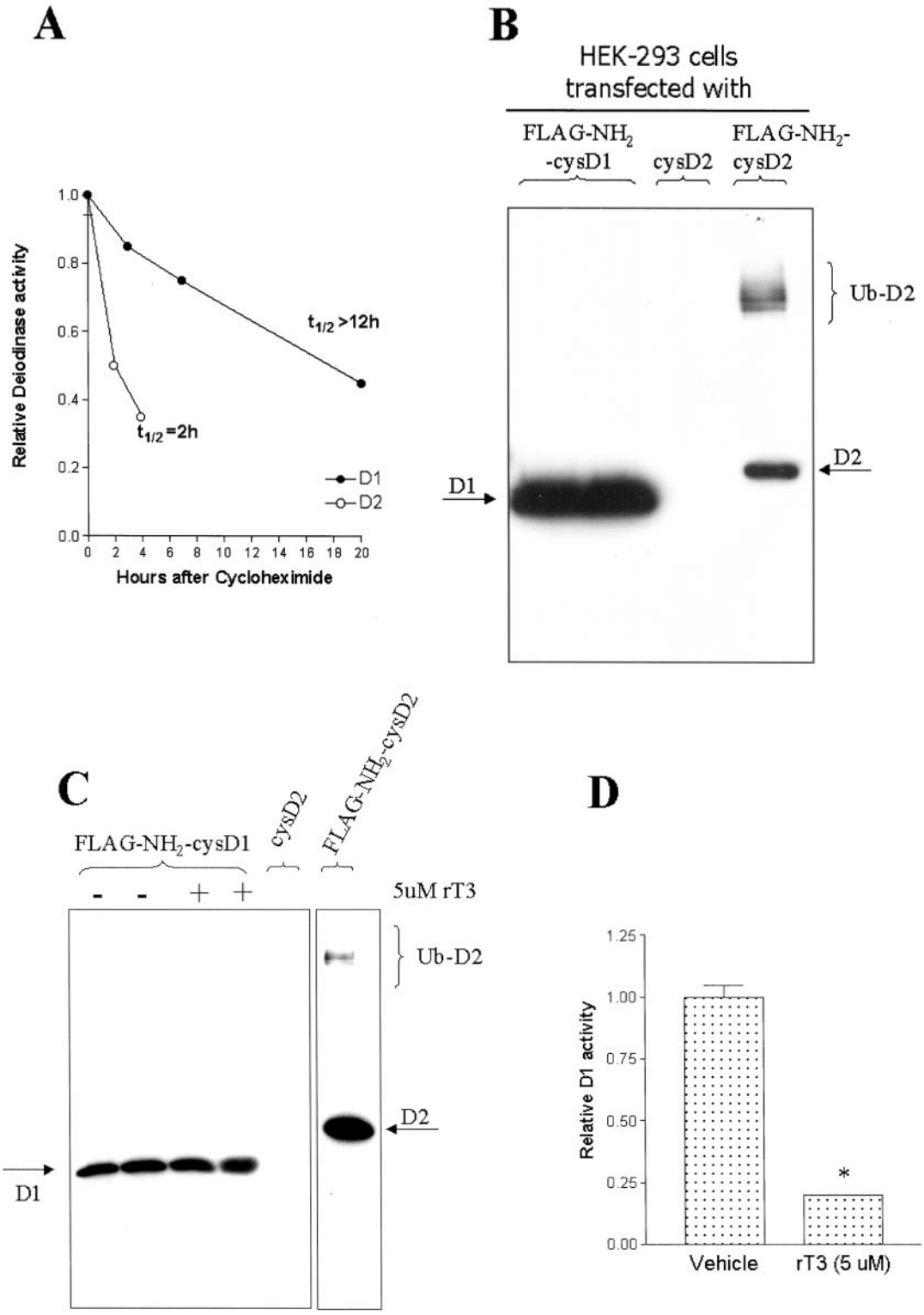

Fig. 7. Transiently Expressed cysD1 Is Not Ubiquitinated in HEK-293 Cells

A, HEK-293 cells transiently expressing either FLAG-NH_2-cysD1 or FLAG-NH_2-cysD2 were treated with 100 μM CX, harvested at the indicated times, and processed for D1 or D2 activities. B, Similarly transfected cells processed for Western blot with anti-FLAG antibody after 12% SDS-PAGE. Cells transiently expressing cysD2 were used as a negative control. C, HEK-293 cells transiently expressing FLAG-NH_2-cysD1 were treated with 5 μM rT_3. Twenty four hours later the cells were processed by Western blotting with anti-FLAG antibody (C) or for D2 activity (D). Cells transiently expressing FLAG-NH_2-cysD2 or cysD2 were used as controls. *, $P < 0.05$ *vs*. vehicle-treated cells.

ates ubiquitination. This would explain why an oxidizing agent such as diamide irreversibly inactivates D2 (20). Indeed, such a redox-sensitive mechanism has been described for the ubiquitination and proteasomal degradation of hypoxia-inducible factor 1α, involved in the activation of the erythropoietin gene (21). However, a catalytically inactive D2 mutant, in which alanine was substituted for the Sec in the active center of the enzyme (alaD2), retains the typical D2 short half-life while it is refractory to the substrate-induced acceleration of its proteolysis (11). If the redox state of the active center plays a role in accelerating ubiquitination it must be limited to the latter process.

The results with D1 provide an important contrast suggesting that simply deiodinating an iodothyronine does not accelerate ubiquitination of a deiodinase. The present results confirm previous data that D1 activity is also decreased by exposure to substrate (20), but our results show that this does not involve D1 ubiquitination (Fig. 7). Rather, the inactivation process is most likely the consequence of oxidation of the active center Se that requires time until the intracellular environment returns it to the reduced state, as originally proposed (22). The finding that D1 is not ubiquitinated can also explain its much longer half-life (Fig. 7). Taken together, these results indicate that T_4 to T_3 conversion by D2 is more tightly regulated than that by D1. Whether this is due to differences in protein structure or in subcellular localization between the two selenodeiodinases remains to be determined.

In summary, we propose that newly synthesized D2 (D2-Se$^-$) exists in the ER membrane in a dynamic equilibrium with its ubiquitinated (inactive) derivative Ub-D2-Se$^-$ (Fig. 8). The latter may be deubiquitinated by isopeptidases, thus reactivating it, or it may enter proteasomes for irreversible degradation. T_4, the principal substrate of D2 [or rT_3 (10, 11)], induces poorly understood changes in the enzyme forming D2-Se, thus accelerating the ubiquitination process, shifting the equilibrium toward Ub-D2-Se. Whether Ub-D2-Se can be reactivated *in vivo* is not clear. The critical change induced in D2-Se$^-$ by T_4 constitutes a posttranslational control process regulating the rate of T_4 activation. Such a mechanism is especially well suited to T_4 since its 7-day plasma half-life in humans precludes minute-to-minute variations due to changes in secretion via feedback at the hypothalamic-pituitary level. However, given the advantages of local intracellular control of hormone activation, it would not be surprising if other hormone systems are regulated in a similar fashion.

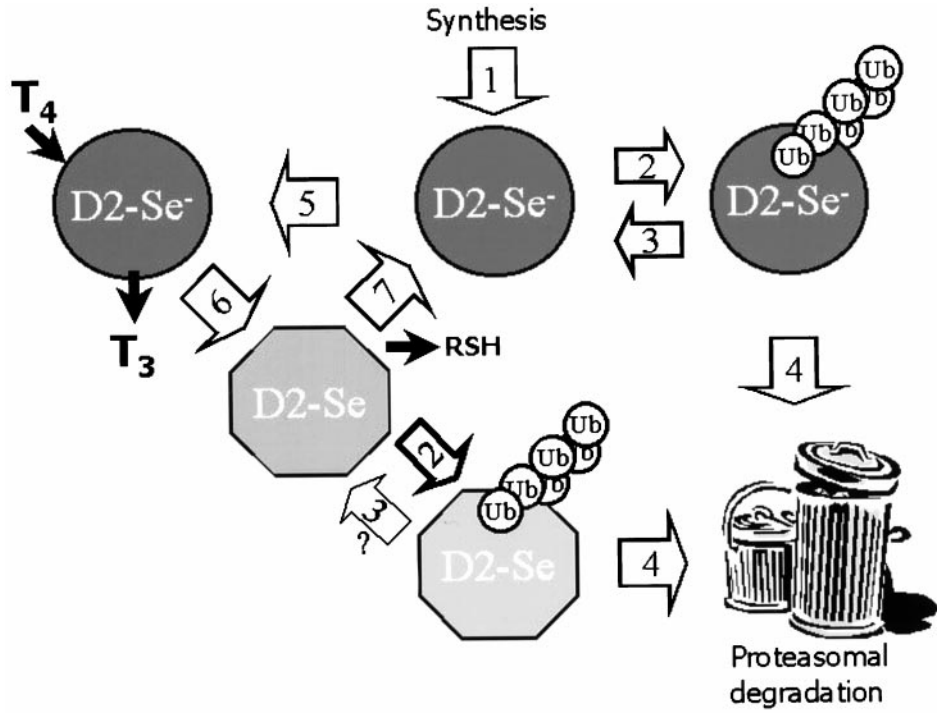

Fig. 8. Proposed Model of D2 Ubiquitination and Degradation by Proteasomes

D2 (*dark circle*) is synthesized (1) and remains as a resident protein in the ER. During its normal turnover it is ubiquitinated (2). Deubiquitination by isopeptidases (3) is possible particularly under conditions where the proteasomal degradation (4) is impaired. Catalysis (5) results in oxidation of the Se in the active center (*hexagon*). This oxidation or another structural change caused by catalysis (6) accelerates ubiquitination (2) and eventual degradation (4). Alternatively, an intracellular reducing agent may regenerate the active form of D2 (7) after deubiquitination (3), although there is currently no evidence to establish the presence of this pathway.

MATERIAL AND METHODS

Reagents

MG132, CX, rT_3, and T_4 were obtained from Calbiochem (La Jolla, CA). The iodothyronines (Sigma , St. Louis MO) were dissolved in 40 mM NaOH and the other drugs were dissolved in dimethylsulfoxide (DMSO). Pansorbin was from Calbiochem. Outer ring-labeled T_4 (specific activity: 4400 Ci/mmol) was from NEN Life Science Products, Inc. (Boston, MA). $Na_2[^{75}Se]O_3$ was kindly provided by the University of Missouri Research Reactor, courtesy of Drs. Marla Berry and Dolph L. Hatfield. All other reagents were of analytical grade.

Preparation of D1- and D2-Expressing Plasmids and Mutagenesis

All constructs were cloned into the D10 mammalian expression vector (23). Wild-type D2 constructs contained the SelP SECIS element (24). Overlap-extension PCR was used to produce D2 mutants where the Sec 133 was replaced by Cys (cys-D2), as described previously (11), and subcloned into the same vector. A D1 mutant in which Cys was replaced by Sec (G5; cysD1) was described previously (12).

Epitope-tagged D2 proteins were created using the eight-amino acid FLAG sequence (Sigma). The NH_2-FLAG-cysD2 (Met-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys Leu-Ala-Met) was generated by the Bp84-Bp85 oligonucleotides [the former contained an *Nco*I site, while the latter contained a *Sal*I site (*lower case*)] by Vent PCR on a hD2 Cys template. (Bp84 sense; 5′-catgccATGG GCATCCTCAG CGTAGACTTG CTGA; Bp85 antisense: 5′-ttccgcggcc gctatggccg acgtcgacTT AACCAGCTAA TCTAGTTTTC TTTCATCT). The resulting fragment was cut by *Nco*I/*Sal*I and cloned into the corresponding sites of the D10 vector, which also contained a 5′-*Eco*RI site and a strong Kozak consensus (-3A) sequence with an in-frame *Nco*I site at the 3′-end of the FLAG sequence (Fig. 3A). This construct does not contain a SECIS element so that translation of the NH_2-FLAG-cysD2 terminates at the second UGA codon, the eighth codon from the COOH end of the native D2 protein. Previous studies showed no effect of this truncation on enzyme activity (25). The COOH-FLAG-cysD2 Cys mutant was generated by the Bp97-Bp95 oligonucleotides [the former contained an *Eco*RI site, while the latter contained an *Xba*I site (*lower case*)] by Vent PCR on a hD2 Cys template. (Bp97 sense: 5′-ggaattcatt ATGGGCATCC TCAGCGTAGA CTTGCTGATC A; Bp95 antisense: GCTCTAGAtt acttgtcgtc atcgtccttg tagtcACCAG CTAATCTAGT TTTCTTaCAT CTCTTGCT). The sense oligo contained the same Kozak consensus as the NH_2-FLAG-cysD2 construct, to ensure the same rate of translational initiation. We also replaced the second Sec codon (UGA) with Cys (UGU) using the Bp95 antisense oligo, to ensure uniform translation (Fig. 3B). Both FLAG-cysD2 constructs were catalytically active.

The NH_2-FLAG-cysD1 mutant was generated by the Bp92-Bp93 hD1 oligonucleotides on a rat Cys mutant template (G5), incorporating *Nhe*I and *Sal*I restriction sites (*lower case*) at the 5′- and 3′-ends of cysD2, respectively (Bp92 sense; ctagctagcc ATGGGGCTGC CCCAGCCAGG GCTGTGGCTG A; Bp93 antisense: ttccgcggcc gctatggccg acgtcgacTT AACTGTGGAG CTTTTCCAGA ACAGCACGA). The fragment was cut by these enzymes and cloned between the corresponding sites of a version of the above described NH_2-FLAG D10 fusion vector, containing a unique *Nhe*I site 3′ to the FLAG sequence (Fig. 3C). The resulting protein is a rat NH_2-FLAGcysD1 containing Pro, Pro, and Gly in positions 4, 6, and 7, respectively, as in the human wild-type D1. Its COOH terminus contains His and Ser in positions 248–249 and is followed by a stop codon, making it a 29-kDa protein. The protein was catalytically active. None of the FLAG-tagged Cys mutant deiodinase fusion proteins contained a SECIS element. The accuracy of the construction was confirmed by sequencing.

Procedures for Transfections

D1 and D2 were transiently expressed by introducing expression vectors containing the respective cDNAs into human embryonic kidney epithelial cells (HEK-293) or Chinese hamster ovary (CHO) ts20 cells. To obtain uniform expression in all plates in an experiment, we used the following pair-plating approach to the transfection. Cells were initially plated in 60-mm dishes and grown until confluence in DMEM supplemented with 10% FBS. Plasmid DNA was precipitated in ethanol and then redissolved in 0.25 M $CaCl_2$ in HEPES buffer and equal amounts of suspension added to the pair of plates. Ten micrograms of D10 vector containing D1 or D2 cDNAs were combined with 4 μg of a D15 vector and 3 μg of TKGH plasmid per plate. Cells and plasmid DNA were allowed to stand for 20–30 min at room temperature and subsequently incubated at 37 C. hGH was measured in the media 48 h later as a monitor for transfection efficiency. Differences in hGH expression in cells transfected with the same DNA precipitate were $<5\%$.

^{75}Se incorporation and D2 IP

These procedures were performed as previously described (11). Briefly, transfected ts20 cells were labeled *in vivo* with 4–6 uCi of $Na_2[^{75}Se]O_3$/dish on day 2 after transfection in the presence of DMEM supplemented with 10% FBS. On day 3, the cells were lysed for 2–3 h at 4 C using a lysis buffer [1% Triton X100, 1% bovine hemoglobin, 1 mM iodoacetamide, 0.2 U aprotinin/ml, 1 mM phenylmethylsulfonyl fluoride (PMSF) in TSA buffer (0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.025% NaN_3)] 1 ml/dish. After centrifugation of the lysate at 1,000 rpm for 5 min, each 1 ml supernatant was incubated for 12–24 h at 4 C with preimmune rabbit sera to a final dilution of 1:100. One hundred microliters of a 10% Pansorbin suspension were then added per tube and incubated under slow agitation for 20 min at 4 C. After centrifugation at 1,000 $\times$ *g* for 7 min, the supernatants were incubated for 24–48 h at 4 C with a D2 rabbit polyclonal antibody [No. 85254 (11)] to a final dilution of 1:100. This antibody was generated against a synthetic peptide SRSKSTRGEWRRMLTSEGLRC (residues 52–72) selected from the human D2 protein. Immunoprecipitates were obtained after the addition of 100 μl of a 10% Pansorbin suspension and centrifugation at 1,000 rpm for 7 min. The pellets were then washed four times with a dilution buffer (0.1% Triton X 100, 0.1% bovine hemoglobin in TSA) and then washed once in TSA buffer and once with 0.05 M Tris-HCl, pH 6.8. Pellets were then heated at 95 C for 7 min in sample loading buffer and spun at top speed for 5 min, and 30 μl of the supernatants were used for analysis by SDS-PAGE.

Western Blots of Epitope-Tagged D2

HEK-293 or ts20 cells transiently expressing the various constructs were scraped, washed in PBS, suspended in lysis buffer (0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.25 M sucrose, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin), and sonicated for 3–4 sec. In one experiment cell sonicates were centrifuged at 2,500 $\times$ *g* for 10 min, and the supernatant was spun at 100,000 *g* for 1 h to separate microsomes from cytosol. Protein concentration was measured as described by Bradford (26), and 20–40 μg were analyzed by 7.5% or 12% SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane (Immobilon, Millipore Corp., Bedford, MA). The blots were probed with an anti-FLAG M2 antibody (1:3333, Sigma) or with a polyclonal anti-Ub antibody (1:1000, Chemicon, Temecula CA), or both. In the latter case,

Ub detection was followed by stripping and exposure to the same FLAG detection. Samples processed for the Ub antibody were previously purified on M2-anti-FLAG affinity agarose (Sigma). The Western blot was carried out using the Chemiluminescence Kit of Roche Molecular Biochemicals (Indianapolis IN), according the instructions of the manufacturer.

Statistical Analysis

Data are presented as mean ± SD throughout the studies. Student's *t* test was used for comparative analysis. Five percent was the level of significance required to reject the null hypothesis.

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