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Substrate-Induced Down-Regulation of Human Type 2 Deiodinase (hD2) Is Mediated through Proteasomal Degradation and Requires Interaction with the Enzyme's Active Center*

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ABSTRACT

Type 2 iodothyronine deiodinase (D2) catalyzes the first step in thyroid hormone action, the deiodination of T_4 to T_3 . Endogenous D2 activity is posttranslationally regulated by substrate that accelerates its degradation through the ubiquitin-proteasome pathway. To understand how D2 activity correlates with D2 protein during its normal decay and rT_3-induced down-regulation, HEK-293 cells, transiently expressing human D2, were labeled with $Na^{75}SeO_3$ and then treated with 100 μM cycloheximide (CX), 30 nM rT_3, and/or 10 μM MG132, a specific proteasome inhibitor, for 2–4 h. D2 protein and enzyme activity changed in parallel, disappearing with a half-life of 2 h in the presence of CX, or 1 h when CX + rT_3 were combined. Treatment with MG132 blocked these effects. We created selenocysteine (Sec) 133 to cysteine (Cys) or alanine (Ala) D2 mutants, without changing Sec 266. The CysD2 activity and protein levels were also parallel, with a similar half-life of approximately 2 h, whereas the rT_3-induced D2 down-regulation required approximately 1000-fold higher rT_3 concentration (30 μM) due to a proportionally higher Michaelis constant of CysD2. In similar experiments, the AlaD2 mutant retained the short half-life but was not catalytically active and not susceptible to rT_3-accelerated degradation. We conclude that substrate-induced loss of D2 activity is due to proteasomal degradation of the enzyme and requires interaction with the catalytic center of the protein. (*Endocrinology* **141:** 1127–1135, 2000)

THE TYPE 2 iodothyronine deiodinase (D2) (M_r ~31 kDa) is the most recently cloned member of the deiodinase family, which consists of three integral membrane selenoproteins, types 1, 2, and 3 iodothyronine deiodinase (D1, D2, and D3). Each of these contains the rare amino acid selenocysteine (Sec) in the highly conserved active center (1–5). The presence of Sec accounts for many of the biochemical properties that characterize D2 catalyzed deiodination, including high catalytic efficiency and substrate affinity (6).

D2 expression is tissue specific and can be regulated by transcriptional and posttranscriptional mechanisms. For example, the expression of the 7.5-kb D2 messenger RNA (mRNA) found in human brain and pituitary gland has recently been shown to be inversely proportional to thyroid status (7–9). In the brown fat, D2 mRNA is markedly increased by the adrenergic stimulation during exposure of rats to cold (7) and in the pineal gland the nocturnal increase in D2 activity is preceded by the increase in its mRNA (10). At the posttranslational level, it has been known for a number of years that D2 activity is rapidly down-regulated by iodothyronines (11–18). The substrate-induced down-regulation of D2 activity is apparently mediated by posttranslational mechanisms rather than the rate of enzyme synthesis because it occurs in the presence of inhibitors of transcription or translation (15, 18). The isolation of D2 mRNA made it possible to compare the effect of rT_3 on D2 mRNA and activity in pituitary tumor cells. Kim *et al.* found that exposure of GH4C1 cells to 50 or 100 nM rT_3 caused a time-dependent 80–90% reduction in the D2 activity but no change in D2 mRNA (19).

The mechanism for substrate-induced inactivation of D2 has been studied in both rat pituitary tumor cells and primary cultures of rat glial cells. In GH3 pituitary cells, D2 activity has a half-life of 50 min that is reduced to 26 min by rT_3 (18). Other D2 substrates (T_4 and iopanoic acid) have similar effects. Acceleration of degradation was enhanced by diamide which depletes the cell of reduced thiols and D2 was regenerated more rapidly in cells exposed to DTT (18), suggesting that D2 inactivation is accelerated by oxidation of the active site by substrate. In hypothyroid rat glial cells, the D2 activity is 2- to 5-fold increased over the levels found in cells grown with normal serum. The addition of cycloheximide (CX) or rT_3 rapidly decreases D2 activity confirming the short half-life and the substrate-induced down regulation of D2. In these cells it was also found that D2 degradation is not affected by lysosomotrophic agents such as chloroquine or NH_4Cl but was partially blocked by ATP-depletion (20).

The rapid turnover rate of D2 and the observation that ATP-depletion partially blocks loss of D2 activity prompted us to investigate the role of the ubiquitin (Ub)-proteasome pathway in D2 degradation. The proteasome is a large com-

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plex of proteases (26S) present in all eukaryotes to which ubiquitination targets proteins for degradation (21). Indeed, we have found that in rat pituitary tumor cells the short half-life of endogenous D2 is the result of its degradation by the Ub-proteasome system. Enzyme activity in the presence of CX was sustained for several hours by MG132 or lactacystin, specific inhibitors of the proteasomes. In addition, the substrate (rT_3)-induced reduction of D2 half-life was also blocked in the presence of these proteasome inhibitors (22).

The first goal of the present studies is to substantiate that the short half-life of D2 activity and its down-regulation by substrate are intrinsic properties of the enzyme *per se*, and this can be observed with transiently expressed protein in cells not expressing endogenous D2. If so, then by labeling the protein with ^{75}Se we can determine whether substrate-induced loss of D2 activity is consequent to enzyme degradation in the Ub-proteasome system or to D2 inactivation or some other effect. The second task is to examine the role of substrate interaction with the enzyme during the process of substrate-induced D2 down-regulation by studying transiently expressed mutant D2 proteins in which the Sec-encoding TGA codon at position 133 in the active center of the enzyme has been changed to one encoding cysteine (Cys) or alanine (Ala). The results of such studies will provide the first molecular insights as to the mechanism by which substrate reduces D2 activity.

Materials and Methods

Reagents

MG132 was obtained from the Peptide Institute, Inc. (Osaka, Japan) and dissolved in DMSO. Cycloheximide (CX) and rT_3 were from Calbiochem (La Jolla, CA). CX was dissolved in DMSO and rT_3 in 70% ethanol. Pansorbin was from Calbiochem. Outer ring-labeled [^{125}I]-T_4 specific activity: 4400 Ci/mmol) was from DuPont (Boston, MA). $Na^{75}SeO_3$ 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.

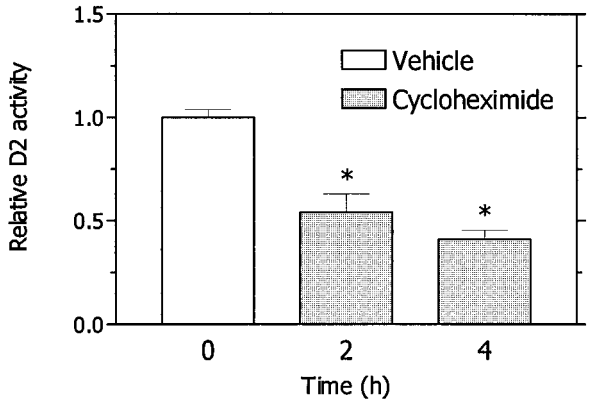

FIG. 1. Disappearance of D2 activity in HEK-293 cells transiently expressing wild-type D2 in the presence of 100 μM CX. Activity is expressed relative to the mean of the vehicle (2–3 μl of DMSO in 2 ml of medium) ± SD in three separate experiments, each performed in triplicate. The D2 activity in the control group was 8.8 ± 4.5 fmol of T_4 deiodinated/mg·min. *$P < 0.05$ *vs.* vehicle.

Preparation of D2 expressing plasmids and their transient expression

A D10 eukaryotic expression vector containing KD2-SelP [K indicates the presence of a Kozak consensus sequence 5′ to the initiator ATG of the human D2 (hD2) coding region and SelP a SECIS element from the SelP gene (23)] was used for transient transfection of human embryonic kidney epithelial cells (HEK-293). Alternatively, mutant D2 complementary DNAs (cDNAs) were prepared in which a Cys or an Ala were substituted for Sec 133. These cDNAs were placed in the same vector.

Mutagenesis

Overlap-extension PCR was used to convert the Sec-encoding TGA codon at position 133 in the hD2 sequence to either TGC (Cys) or a GCA (Ala) codon. A 459-bp *Acc*I fragment containing these mutations was then exchanged for the wild-type fragment in KD2-SelP in the D10 vector (5). These mutant D2 enzymes are referred to as Cys or AlaD2. The Sec residue at position 266 was not altered, thus permitting labeling of Cys or AlaD2 with ^{75}Se-selenocysteine. A version of CysD2 not containing a Sec 266 residue was generated by removing the Xba fragment containing the SelP SECIS element. In this construct, the TGA at 266 becomes a stop codon. The sequences of the mutated cDNAs were verified by manual and automated sequencing. Kinetic studies showed the Ala 133 was inactive and the Sec 133 Cys mutation had an approximately 500-fold increase in the apparent Michaelis constant (K_m) (6).

Studies of D2 transfected cells

The hD2 or mutated proteins were transiently expressed by introducing expression vectors containing the wild-type or mutant D2 cDNA into HEK-293 cells. To obtain uniform expression of D2 in all plates in an experiment, we used the following batch type approach to the transfection for studies on D2 activity. HEK-293 cells grown in T-75 flasks were suspended in 5 ml of PBS (pH 7.3). Transfections were then performed in each batch. Plasmid DNA was precipitated in ethanol and then redissolved in 0.25 M $CaCl_2$ in HEPES buffer and added to each cell batch. Twenty micrograms of D10 vector containing wild-type D2 or mutant D2 were transfected together with 8 μg of a D15 vector in DMEM with 10% FBS. Cells and plasmid DNA were allowed to stand for 20–30 min at room temperature. Transfected cell batches from several T-75s were then pooled and cells seeded in 60-mm dishes. In an alternative approach, HEK-293 cells were initially plated in 60-mm dishes and grown until confluence in DMEM supplemented with 10% FBS. Plasmid DNA was then transfected as CaP precipitates in pairs of plates and incubated for approximately 10 h. Cells from 16–20 plates were then resuspended in PBS, pooled, and seeded again in 60-mm plates to maximize the homogeneity of transfection expression between plates.

Studies on D2 activity

Each experiment was performed with triplicate dishes for each condition. This was done in serum-free DMEM supplemented with 0.1% BSA to reduce nonspecific binding of rT_3. The final concentrations of DMSO and ethanol used to add CX, rT_3, and MG132 were 0.2% and 0.1%, respectively, and were present in all plates as vehicle. At the appropriate time, cells were harvested and D2 activity measured. D2 activity was measured as described previously (22). Briefly, cells were harvested, washed, sonicated briefly in 0.1 M potassium phosphate-1 mM EDTA, pH 6.9 (PE buffer) containing 10 mM DTT and 0.25 M sucrose. Cell homogenates were then assayed for deiodination of freshly purified 2 nM [^{125}I]-T_4. Incubations were carried out for 2 h at 37 C using 300 μg of protein per sample. Protein determinations in duplicate were by Bradford using BSA as standard. D2 activity is reported as fmol of T_4 deiodinated/mg·min.

Production of anti-D2 antisera

We examined the amino acid sequence, surface probability, antigenic index, α, β, and turn regions of D2 and selected four peptide sequences that were synthesized and combined with KLH by Research Genetics, Inc., Huntsville, AL. The KLH-peptides were emulsified by mixing with an equal volume of Freund's adjuvant and injected into 3–4 sc dorsal

sites of 3- to 9-month-old New Zealand white rabbits (Research Genetics, Inc.), for a total volume of 1 ml (0.1 mg of peptide) per immunization. Bleedings were performed before immunization and 4, 8, 10, and approximately 14 weeks afterwards (see Fig. 5B). Boost injections were given after 2 and 6 weeks. The antipeptide antibody titer was determined by ELISA with free peptide on the solid phase (1 μg/well). Only the antisera with the highest titers from each rabbit were used.

^{75}Se incorporation studies and D2 IP

Transfected HEK-293 cells were labeled *in vivo* with 4–6 μCi 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 X-100, 1% bovine hemoglobin, 1 mM iodoacetamide, 0.2 U aprotinin/ml, 1 mM PMSF in TSA buffer (0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, 0.025% NaN_3)] 0.5–1 ml/dish. After centrifugation of the lysate at 1,000 rpm for 5 min each 0.5–1 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 one of several D2 rabbit antisera (see above) at a final dilution of 1:100. Immunoprecipitates were obtained following 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), then 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, spun at top speed in a microfuge for 5 min, and 30 μl of the supernatants analyzed.

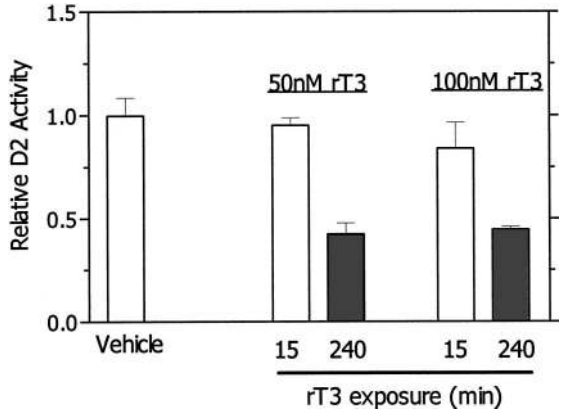

FIG. 2. Effects of time and rT_3 concentration on transiently expressed D2 activity in HEK-293 cells. Activity is expressed relative to the mean of the vehicle (2–3 μl of 40 mM NaOH in 2 ml of medium) $\pm$ SD of two separate experiments, each performed in duplicate. The D2 activity in the control group was 8.8 $\pm$ 4.5 fmol of T_4 deiodinated/mg·min. *$P < 0.05$ *vs.* vehicle and 15-min time point.

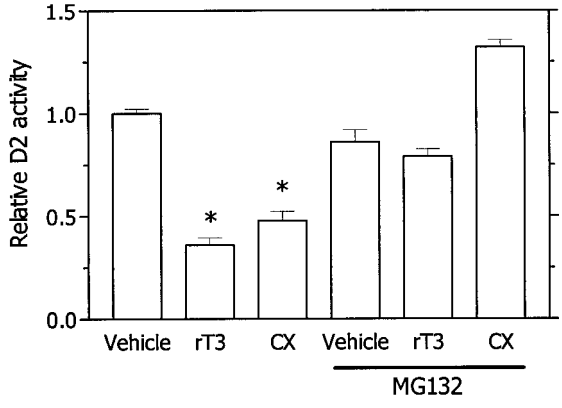

FIG. 3. Effects of rT_3, CX and/or MG132 on D2 activity in HEK-293 cells transiently expressing hD2. MG132 (10 μM) was introduced 10 min before CX (100 μM). rT_3 (100 nM) was introduced simultaneously with CX. Treatments lasted 2 h. Results are expressed as percentage of the activity in vehicle-treated cells $\pm$ SD of two to four different experiments, each performed in triplicate. D2 activity in vehicle was 8.5 $\pm$ 1.9 fmol of T_4 deiodinated/mg·min. *$P < 0.05$ *vs.* vehicle.

Statistical analysis

Results of D2 assays were expressed as mean $\pm$ SD of the plates studied for each condition (n = 6–12 replicates) in three separate experiments. Because there were variations in basal D2 activities among various groups of cells in different experiments (from 5.0 $\pm$ 0.5 to 14.2 $\pm$ 1.5 fmol T_4 deiodinated/mg·min for wild-type D2 and from 0.3 $\pm$ 0.02 to 0.8 $\pm$ 0.12 fmol T_4 deiodinated/mg·min for the CysD2 mutant), we normalized results for each experiment to the mean of the control values for that experiment. A one-way ANOVA with the Newman-Keuls test for multiple comparisons was used to assess the statistical significance of a given treatment. $P < 0.05$ was considered significant.

Results

Transiently expressed D2 in HEK-293 cells is inactivated in proteasomes

The first experiments characterized the pathway of degradation of transiently expressed D2. When CX was added to cells transfected 24 h earlier with D2 cDNA, D2 activity was decreased to approximately 50% 2 h later, indicating a half-life of approximately 2 h. Over the next 2 h, the activity fell another 30%, indicating that the decrease in D2 activity was not log linear over 4 h in the presence of CX (Fig. 1). Transfected HEK-293 cells were next incubated with 50 nM rT_3 for 15 or 240 min and processed for D2 activity. After 4 h incubation with 50 or 100 nM rT_3, D2 activity was approximately 45% of control. There was no effect of a 15-min incubation, indicating that the loss of activity is time dependent and not explained by dilution of the T_4 substrate by the rT_3 added to induce the effect (Fig. 2). The minimum rT_3 con-

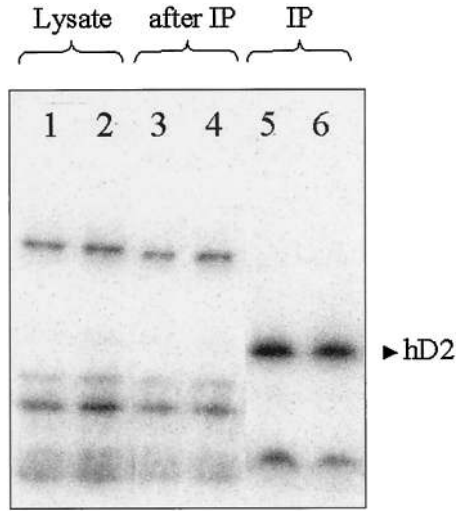

FIG. 4. IP of D2 protein from ^{75}Se-labeled HEK-293 cells transiently expressing hD2. HEK-293 cells were transfected with hD2 and labeled with ^{75}Se. IP was carried out as described in *Materials and Methods* using anti-NH2 antiserum no. 85254 at a dilution of 1:100. Five microliters of total lysate (1 ml; lanes 1–2) or IP supernatant (1 ml; lanes 3–4) were loaded on each lane. Sixty-five percent of the immunoprecipitates were loaded on lanes 5–6.

centration required to obtain an effect was 20 nM. T_4, 50 nM, also caused a significant decrease in D2 activity, but a formal dose-response comparison between rT_3 and T_4 was not performed.

We next evaluated the effects of MG132 on the CX and rT_3-induced decrease in D2 activity. Cells transiently expressing wild-type D2 were incubated with CX or rT_3 in the presence or absence of MG132, a specific inhibitor of the Ub-proteasome pathway (Fig. 3). Incubation with 100 μM CX for 4 h reduced D2 activity by approximately 50%, an effect that was completely blocked when MG132 was also present in the incubation medium. Furthermore, the approximately 60% reduction in D2 activity during exposure to 100 nM rT_3 was also blocked by MG132. Altogether, the data indicate that the D2 transiently expressed in HEK-293 cells has similar characteristics to the endogenous D2 in GH4C1 cells.

Identification and quantification of D2 by IP

The decrease in D2 activity induced by substrate and by blockade of protein synthesis could be due to degradation of D2 *per se* or to loss of some other protein required for enzyme function. To clarify this issue, we needed a method to identify D2 independently of its catalytic activity. For these experiments, we took advantage of the presence of the two Sec residues at position 133 and 266 in D2, and the availability of ^{75}Se as $Na^{75}SeO_3$. We also prepared rabbit antisera directed against four different D2 epitopes. One day after transfection of D2 expressing plasmid, ^{75}Se was added to each plate, and after another day the cells were harvested using lysis buffer and subsequently processed for IP. Radioautography of the lysates showed at least 6 ^{75}Se-labeled bands, all of which were present in control nontransfected cells except a poorly visualized band at approximately 31 kDa (Fig. 4). This band, the predicted size of hD2, was substantially decreased by IP with a D2 antiserum and was enriched in the precipitate of transfected cell lysates using eight different antisera against four different D2 epitopes, two from the NH_2-terminal portion of the protein and two from the COOH- terminal region (Figs. 4 and 5). The CysD2 ΔXBa construct will not encode a seleno-D2 protein because the SECIS element has been deleted and therefore serves as a negative control (Fig. 5). The only nonD2 related ^{75}Se-labeled

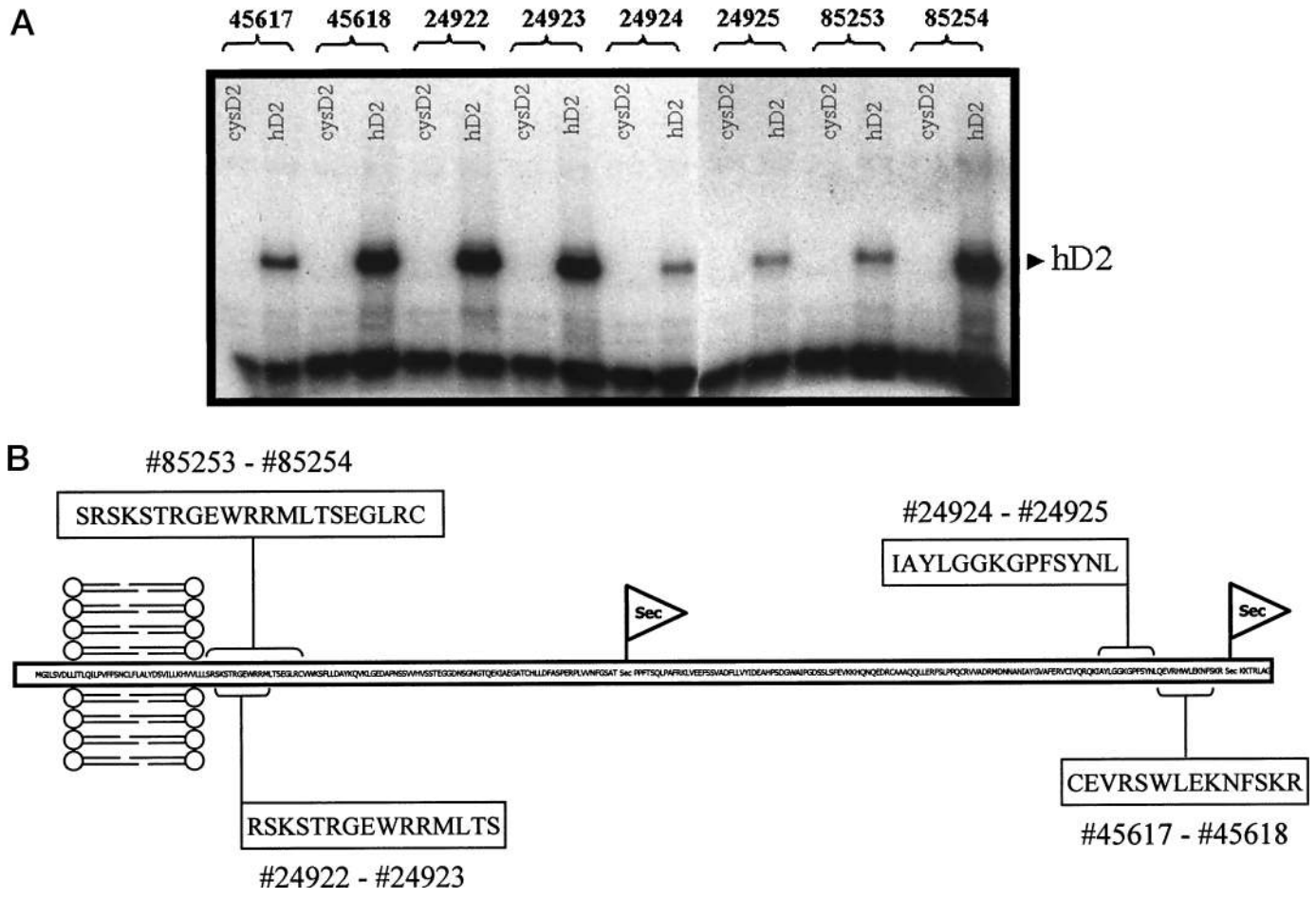

FIG. 5. D2 antisera used for IP of D2 protein from ^{75}Se-labeled HEK-293 cells transiently expressing hD2. A, Hypothetical representation of D2 protein and its relationship with the ER membrane. The Sec amino acids are flagged. The peptide sequences were used to immunize rabbits and generate antisera used for D2 IP. The numbers under each peptide sequence identify each antiserum. B, HEK-293 cells were transfected with hD2 or CysD2ΔXba, a D2 mutant construct, which contains no SECIS element and in which Cys was substituted for Sec 133. All plates were labeled with ^{75}Se. The autoradiograph of the 12% SDS-PAGE indicate the specific (hD2) and nonspecific (CysD2) precipitation of ^{75}Se-proteins by the eight antisera. All antisera were used at a 1:100 dilution.

protein in the immunoprecipitated material from both control and D2-transfected cells was one of about 15 kDa, which is nonspecific (Fig. 4). These results establish the identity of the 31-kDa protein as hD2.

We then used this tool to determine if the changes in D2 activity induced by CX or rT_3 were accompanied by equivalent changes in the amount of D2 protein. In these experiments, pairs of cell plates were transfected with wild-type D2, labeled with ^{75}Se, and 24 h later treated with 100 μM CX, 50 nM rT_3, and/or 10 μM MG132 for 4 h, exactly as for the activity experiments. The cells were immediately lysed and processed for IP using the anti-NH_2-terminal or anti-COOH-terminal antisera (Fig. 6). The first two pairs of lanes show that rT_3 or Cx causes an approximately 50% reduction in ^{75}Se-D2 protein, in agreement with their effects on D2 activity. Treatment with MG132 blocked both the CX- and rT_3-induced loss of D2 protein, again in parallel with effects on D2 activity (see Fig. 3: pairs 1 *vs.* 3 and 2 *vs.* 4). The pairs in lanes 5 show that MG132 also increases the basal D2 as would be expected if it blocked D2 degradation. This phenomenon is seen with both antisera. These data establish that the loss of D2 activity following CX or rT_3 is due to D2 degradation in proteasomes.

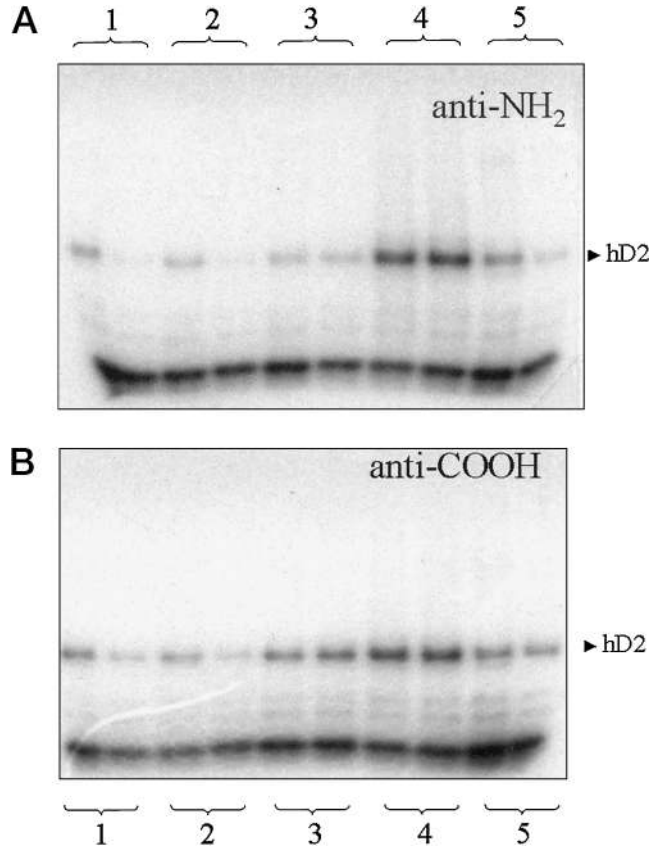

FIG. 6. Effects of rT_3, CX and/or MG132 on ^{75}Se-D2 in ^{75}Se-labeled HEK-293 cells transiently expressing hD2. Pairs of cell plates were transfected with wild-type hD2 and labeled with ^{75}Se. Indicated treatments lasted for 2 h and were performed as in legend to Fig. 3. IP was carried out as described in *Materials and Methods* using anti-NH2 antiserum no. 85253 or anti-COOH antiserum no. 45618. For each pair of cell plates, the intensities of the D2 bands were quantified by densitometry and the ratios (treatment/control) are as follows: Anti-NH2 terminal—Pair 1, 0.35; Pair 2, 0.48; Pair 3, 1.1; Pair 4, 1.1; Pair 5, 2.6. Anti-COOH terminal—Pair 1, 0.48; Pair 2, 0.66; Pair 3, 1.3; Pair 4, 1.1; Pair 5: 1.7.

Correlation between D2 enzyme activity and D2 protein in a batch transfection system

We next took advantage of a batch transfection system to compare the changes in D2 protein with D2 activity in a pool of cells transiently expressing D2. Addition of CX for 2 h reduces D2 activity by approximately 40% (Fig. 7A). The fall in D2 activity was paralleled by a similar decrease in ^{75}Se-D2 protein as measured by IP confirming it is due to D2 degradation (Fig. 7B). When MG132 was added with CX, the loss of both activity and D2 protein were blocked proportionally. This result also implies that D2 protein accumulating in the presence of MG132 is enzymatically intact. However, because most of the ^{75}Se-D2 is 31 kDa (but not heavier), it suggests that the Ub-D2 pool is small probably because D2 is constantly and rapidly deubiquitinated by the Ub-isopeptidases present in most cells. When rT_3 was added in the presence of CX, D2 activity decreased even more (by approximately 50%), though the ^{75}Se-D2 level was only slightly affected. MG132 blocked this effect, the D2 activity decreasing by only 10–15%, with a similar modest change in D2 protein. Altogether, the data indicate the D2 protein *per se* is degraded in proteasomes, and its degradation is accelerated by exposure to rT_3.

An hD2 containing an Sec 133 to Cys mutation is also degraded by proteasomes and responds to substrate

The Sec133Cys substitution (CysD2) was created by PCR mutagenesis and placed in the D10 eukaryotic expression vector. Importantly, the Sec at position 266 was not changed, and the SECIS element remained intact. This allows ^{75}Se labeling of the transiently expressed CysD2. Kinetic studies showed the apparent K_m for rT_3 and T_4 of this mutant D2 was approximately 500-fold higher than for the wild-type as discussed elsewhere [(6); data not shown]. The same type of study as shown in Fig. 7, comparing activity with ^{75}Se-D2 was performed using either 30×10^{-9} M rT_3 or 30×10^{-6} M rT_3, the latter in light of much higher K_m of the CysD2 mutant. Incubations with CX or rT_3 were for 4 h, and the results are shown in Fig. 8. Basal levels of activity for the CysD2 mutant were 0.61 $\pm$ 0.25 fmol/mg·min (Fig. 3), approximately 10 times lower than basal levels for wild-type D2. The half-life of the CysD2 activity in the presence of CX was again about 2 h. There was no effect of the lower dose of rT_3 (data not shown), but incubation with 30×10^{-6} M rT_3 decreased D2 activity by approximately 40%. Figure 8A also shows that, as with the wild-type D2, incubation with 10 μM MG132 increased basal CysD2 activity. The peptide aldehyde also blocked the loss of D2 activity in the presence of CX and the acceleration of D2 disappearance in the presence of rT_3. The changes in D2 activity under these circumstances were mirrored by the IP results (Fig. 8B), indicating that changes in enzyme activity during CX or rT_3 treatment are the result of changes in the quantity of CysD2 protein.

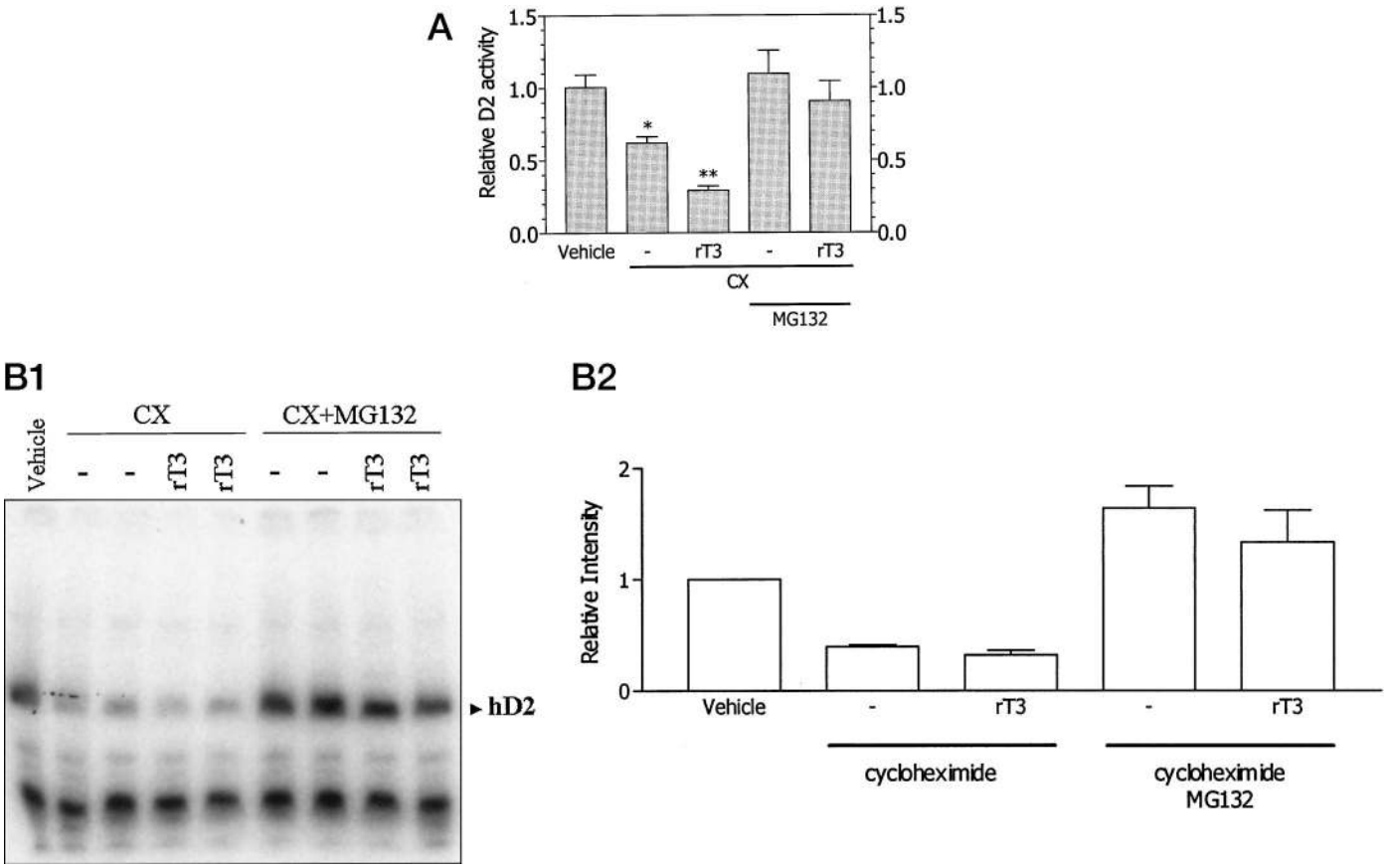

FIG. 7. Effects of rT_3, CX and/or MG132 on D2 activity (A) and ^{75}Se-D2 (B) in HEK-293 cells transiently expressing hD2. Cells were batch transfected with wild-type hD2 and treated as in legend to Fig. 3A.Results are expressed as percentage of vehicle-treated cells in three different experiments. D2 activity in the vehicle group was 12.1 ± 6.6 fmol of T_4 deiodinated per mg·min. *$P < 0.05$ *vs.* vehicle; **$P < 0.05$ *vs.* CX. B, Some plates were labeled with ^{75}Se and processed for IP. The *lower bar graph* indicates the intensity of each D2 band by densitometry.

An Ala 133 mutant of hD2 is also degraded by proteasomes but does not respond to substrate

Interestingly, the AlaD2 mutation, which can be easily visualized by Western blots (data not shown), does not have catalytic activity. Therefore, our analysis was limited to the quantification of the D2 protein by IP. In these experiments, a paired transfection rather than a batch approach was used. Results of a typical experiment show that rT_3 had no effect on D2 protein level but that the rate of disappearance in the presence of CX of AlaD2 protein was identical to wild-type and CysD2 (Fig. 9). This same result was found in two other experiments. Thus, the change in D2 due to the substitution of Ala for Sec blocks both enzymatic activity and substrate-induced degradation of the protein suggesting that there is a causal relationship between these two events.

Discussion

D2 activity is regulated at both the transcriptional and the posttranslational level. Early evidence for the posttranslational regulation derived from studies first in hypothyroid animals and later in cell culture systems showing that the D2 activity has a short half-life and that high affinity substrates for D2, namely rT_3 and T_4, were more potent down-regulators of D2 activity than was T_3 (11–18).

Cloning of the D2 mRNA has allowed demonstration that T_3 will suppress D2 mRNA levels, probably by suppression of transcription (7–9). In some tissues, such as cerebral cortex, the posttranslational effects are more important than are transcriptional changes (7). We recently showed that the decrease in endogenous D2 activity by rT_3 or CX in GH4C1 cells is blocked by MG132 establishing the physiological significance of proteasomes in the posttranslational regulation of D2 activity (22). While this observation is an important step to understanding the mechanism responsible for the short half-life and for the substrate effect on D2, the GH4C1 cell does not lend itself to experiments designed to probe those structural features of D2 that dictate the proteasomal dependence of its degradation and the mechanism(s) by which rT_3 accelerates this. Furthermore, with respect to the primary effect of MG132 to block D2 degradation, we did not have formal proof that it was the degradation of D2 *per se* that was accelerated by rT_3 and blocked by MG132. The present results show that while the t1/2 of transiently expressed D2 activity is about 2 h, twice as long as that in GH4C1 cells (Fig. 1), with respect to the qualitative effects of rT_3 (Fig. 2) and MG132 (Figs. 1 and 3), transiently expressed D2 behaves identically to the endogenous D2 in GH4C1 cells. Furthermore, because it is possible to label the protein with ^{75}Se, we were able to demonstrate a tight correlation between changes

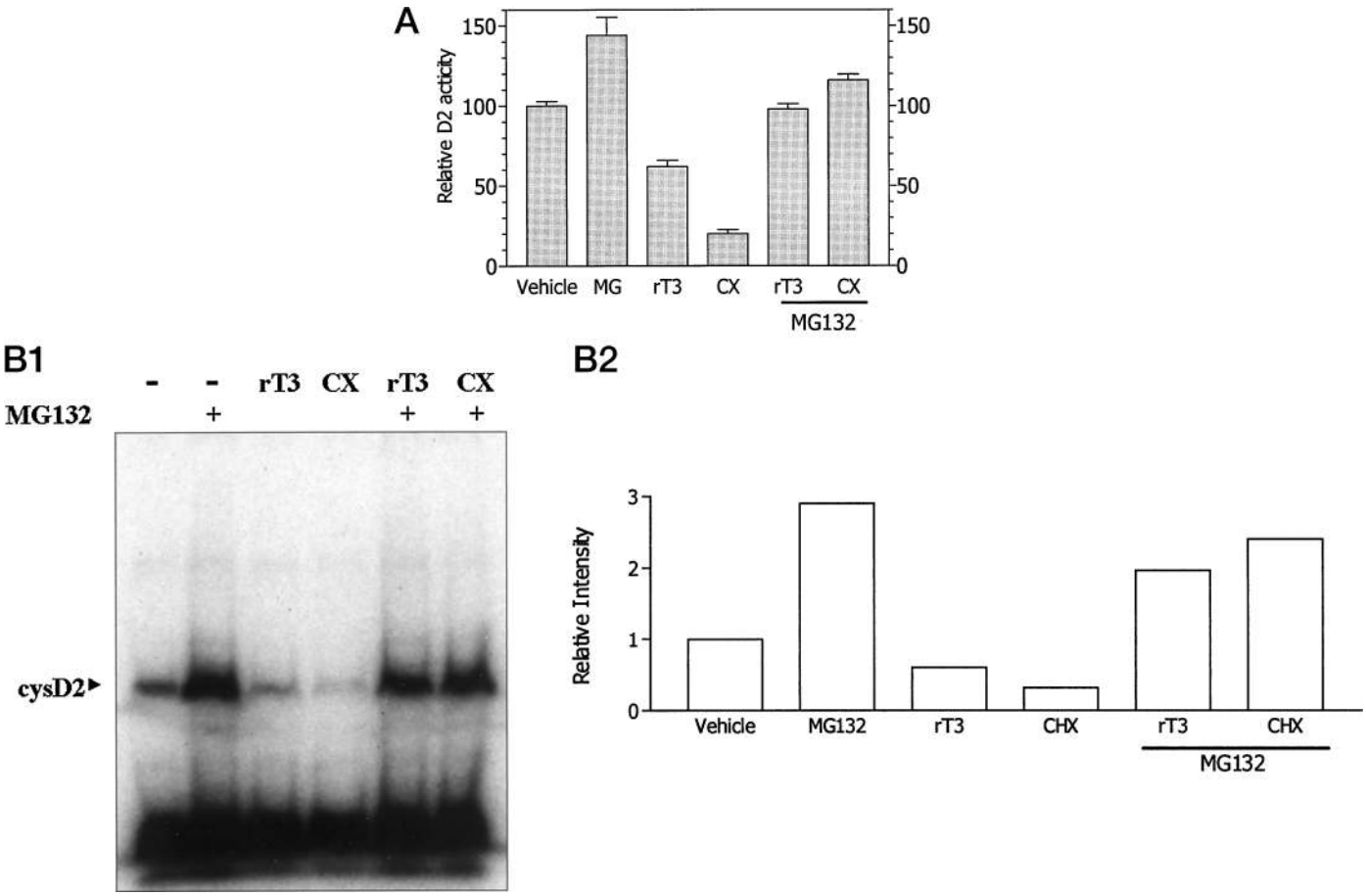

FIG. 8. Effects of rT_3, CX and/or MG132 on D2 activity in HEK-293 cells transiently expressing CysD2. A, MG132 (10 μM) was introduced 10 min before cycloheximide (100 μM). rT_3 (30 μM) was added simultaneously with cycloheximide into the media. Results are expressed as percentage of controls (Vehicle) ± SD of three different experiments, each performed in triplicate. D2 activity in the vehicle group was 0.61 ± 0.25 fmol of T_4 deiodinated per milligrams of protein per minute. $^*P < 0.05$ *vs.* vehicle; $^{**}P < 0.05$ *vs.* rT_3. (B) Some plates were labeled with ^{75}Se and processed for IP. The *lower bar graph* indicates the intensity of each D2 band by densitometry.

in wild-type D2 activity and in the immunoprecipitated ^{75}Se-D2 (Fig. 6).

We can therefore apply this system to begin to probe the one or more mechanisms that confer metabolic instability to D2 and by which substrates such as rT_3 accelerate the proteasomal degradation of this protein. We have first focused on the turnover rate of the D2. Our previous finding that the rapid fall of D2 activity following CX treatment is prevented by MG132 indicates that the normal turnover of D2 is mediated via the Ub-proteasome system. In the present investigation, the close correlation between D2 activity and ^{75}Se-D2 protein confirms that, in fact, the rapid disappearance of enzyme activity is due to proteasomal degradation of the D2 *per se*. This seems to be independent of the substrate-induced degradation of D2 because, as discussed below, the AlaD2 mutation of the enzyme's active center eliminating either selenium or sulfur did not affect the rate of D2 degradation. This is an indication that D2 is an intrinsically unstable molecule that is rapidly targeted by the Ub-system. This depends basically on two steps, conjugation of the substrate with Ub and interaction of the Ub-conjugate with the proteasome. Ubiquitination of proteins occurs only to lysine, of which there are 15 residues in D2 (24). It also may require the presence of degradation signals within the protein molecule to mark it for ubiquitination. Some degradation signals that confer metabolic instability have been reported, *e.g.* N-degron and PEST sequences (25). However, the hD2 sequence does not contain any recognized destabilizing amino acid sequences.

We investigated the active center of the enzyme, changing the Sec to Cys, in effect exchanging S for Se which increases the K_m of the enzyme for rT_3 and T_4 about 500-fold (6) to see if this affects the response of D2 to its substrate. As a consequence, the concentration of rT_3 required to accelerate the degradation of D2 is increased in a parallel fashion (Fig. 7). This suggests that catalysis somehow promotes the degradation of D2. This hypothesis is further supported by the fact that when Ala is substituted for Cys, rT_3 no longer accelerates D2 degradation even though its half-life is not changed by this substitution (Fig. 8). The absence of an effect of rT_3 could be due to the lack of an oxidizable nucleophile (Se or S) in the active center or to a lack of rT_3 binding due to changes in the shape of the binding pocket of D2 secondary to the Ala substitution. Whichever the explanation, the result shows the potential of the transient expression system to address these issues.

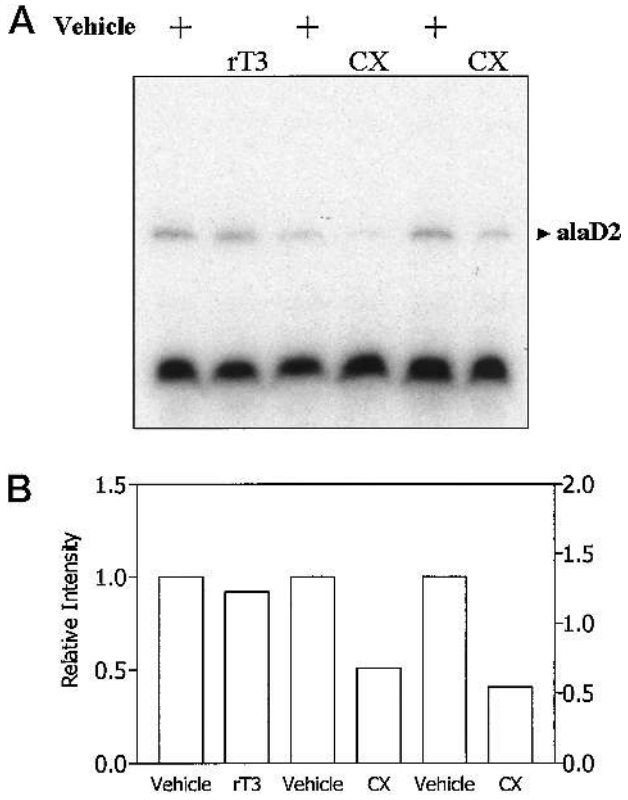

FIG. 9. Effects of rT_3, CX and/or MG132 on ^{75}Se-D2 from ^{75}Se-labeled HEK-293 cells transiently expressing AlaD2. Pairs of plates containing ^{75}Se-labeled HEK-293 cells transiently expressing AlaD2 were treated as in the legend to Fig. 8. After 2-h treatment, cells were harvested and processed for IP with anti-d2 antiserum. The *bar graph* indicates the density of the 75Se-alaD2 band relative to that in the cells of the respective control plate for each pair.

A question raised by the present results with ^{75}Se-D2 is why, when MG132 blocks degradation of ubiquitinated D2, there is no accumulation of a ladder of ^{75}Se-D2-ubiquitin conjugates of increasing molecular size. This apparent paradox has been described in earlier studies of other ubiquitinated proteins and can be explained by the presence of cellular isopeptidases that rapidly deubiquitinate those Ub-protein conjugates not degraded in the proteasomes (26). In cell-free systems, the use of ubiquitin aldehyde (27), which blocks the isopeptidases, allows demonstration of such Ub-D2 conjugates (28). This compound, however, does not cross the cell membrane and therefore could not be used in the present studies. The data in Figs. 4 and 5 showing that D2 can be readily immunoprecipitated by 8 different antisera directed against 4 different D2 epitopes further suggest that there is not a large pool of a poly-ubiquitin D2 conjugate in the cell lysate. In agreement with this is the fact that the activity of D2 is preserved and parallels the changes in ^{75}Se-D2 protein. Another implication of the parallel increase in ^{75}Se-D2 and D2 activity is that the deubiquitinated D2 retains its catalytic activity and was not irreversibly inactivated by interaction with substrate.

In conclusion, these results confirm the effect of rT_3 to accelerate the degradation of D2 via the proteasome in an entirely independent system from the GH4C1 cells. The high transient expression of D2 allows it to be labeled with ^{75}Se and immunoprecipitated. Thus, a precise correlation can be demonstrated between the effects of substrate on D2 activity and D2 protein confirming that it is D2 protein *per se* which is more rapidly degraded during substrate exposure. This system should lend itself to other perturbations of the D2 protein to allow definition of the critical structural elements required for this strikingly rapid nonnuclear mediated effect of rT_3 (and T_4) to regulate the concentration of this enzyme.

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