Degradation of the 74 kDa form of L-histidine decarboxylase via the ubiquitin-proteasome pathway in a rat basophilic/mast cell line (RBL-2H3)

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Abstract L-Histidine decarboxylase (HDC) is a dimer consisting of two identical 53 kDa subunits. On the other hand, the size of HDC deduced from its cDNA sequence is around 74 kDa, indicating that the translated 74 kDa form of HDC is subjected to post-translational processing to generate the 53 kDa form. However, modification of the translated 74 kDa form of HDC in histamine-forming cells is unknown. Here we demonstrate that the 74 kDa form is translated in rat basophilic leukemia cells, followed by conversion to the 53 kDa form, and that the 74 kDa form is a short half-life protein because of the degradation mediated by the ubiquitin-proteasome pathway. Degradation of the 74 kDa form was stimulated in the presence of an ATP-generating system, accompanied by ubiquitination, and inhibited by specific proteasome inhibitors such as ZL_3H and lactacystin. A significant amount of proteasome activity was detected in RBL-2H3 cells.

Key words: Histidine decarboxylase; Ubiquitin-proteasome pathway

1. Introduction

L-Histidine decarboxylase (HDC: EC 4.1.1.22) is a key enzyme in histamine formation in mammals. HDC has been purified and characterized from the soluble fraction of various tissues and cells [1–4]. The purified enzyme comprises a homodimer, composed of two identical 53–55 kDa subunits. On the other hand, cloning of the cDNAs for the enzyme has indicated that the initial translated products are all around 74 kDa [5–7]. We have demonstrated that the recombinant mouse 74 kDa form of HDC, present in the particulate fraction of Sf9 cells, exhibits a low catalytic activity [8], and is cleaved by in vitro incubation with porcine pancreatic elastase to yield a 53 kDa form with full enzymatic activity in the soluble fraction [9]. However, there has been no demonstration of the existence of the 74 kDa form of HDC, and no data on the proteolytic cleavage of the 74 kDa form into the 53 kDa form in vivo. Therefore, the first aim of this study was to investigate the existence of the 74 kDa form and its conversion to the 53 kDa form in a rat basophilic leukemia cell line, RBL-2H3.

Very recently, it has been clarified that the ubiquitin-proteasome system plays an important role in the degradation of short half-life growth regulators such as cyclin and cyclin-dependent kinases, translational activators and inhibitors, and a few enzymes such as tyrosine aminotransferase and ornithine decarboxylase (ODC) [10,11]. ODC is a key enzyme in the regulation of intracellular polyamine levels and cell growth [12–14]. The enzyme is induced by several stimuli [13] and is subject to rapid degradation by the 26S proteasome pathway without ubiquitination [15]. Degradation of a protein through this system involves two successive steps: sequential conjugation of ubiquitin molecules to the target protein and degradation of the protein by the 26S proteasome complex via an ATP-dependent reaction. For the ubiquitin-proteasome system, the putative required sequences are the PEST regions in the target molecule, which are found in extremely short half-life eukaryotic proteins, hydrophilic sequences enriched in Pro, Ser/Thr or Asp/Glu residues flanked by positively charged residues. Detailed structural analysis of the rat HDC cDNA sequence revealed that three PEST regions are present in the translated protein one near the N-terminus and two at the C-terminus of the molecule. In about 21 kDa of the C-terminal region of the 74 kDa form of rat HDC two PEST regions are present, and this region should be cleaved off during post-translational processing [5,7,8]. Thus, we show that the 74 kDa form and not the 53 kDa form was degraded by the ubiquitin/ATP-dependent pathway in RBL-2H3 cells, the degradation being inhibited by proteasome inhibitors.

2. Materials and methods

2.1. Materials

Anti-GST-fusion HDC antiserum was prepared as described previously [16]. The following materials were purchased from the sources indicated: leupeptin and aprotinin from Wako Pure Chemicals (Tokyo, Japan), *n*-acetyl leucyl leucyl norleucinal (ALLN) and *n*-acetyl leucyl leucyl methional (ALLM) from Sigma (St. Louis, MO, USA), carboxybenzyl leucyl leucyl leucinal (ZL_3H), carboxybenzyl leucyl leucyl leucyl 4-methyl-coumaryl-7-amide (z-leu-leu-leu-MCA) and carbobenzoxy leucyl leucinal (ZL_2H) from Peptide Institute (Osaka, Japan), PVDF membranes from Millipore (Tokyo, Japan), mouse anti-ubiquitin monoclonal antibody from Chemicon International Inc. (Temecula, CA, USA), peroxidase-conjugated anti-mouse IgG from Dako (Glostrup, Denmark), ECL Western blot detection reagent from Amersham Life Science (Buckinghamshire, UK), [^{35}S]methionine (1000 Ci/mmol) from Du Pont-New England Nuclear (Boston, MA, USA) and protein A-Sepharose CL-4B and NAP-5

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Abbreviations: HDC, L-histidine decarboxylase; ODC, ornithine decarboxylase; DDC, aromatic L-amino acid decarboxylase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; cDNA, complementary DNA; ATP, adenosine triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); PMSF, phenylmethylsulfonyl fluoride; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; ALLN, *n*-acetyl leucyl leucyl norleucinal; ALLM, *n*-acetyl leucyl leucyl methional; ZL_3H, carboxybenzyl leucyl leucyl leucinal; ZL_2H, carboxybenzyl leucyl leucinal

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columns from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were commercial products of reagent grade.

2.2. Cell culture

RBL-2H3 cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (complete medium) in 5% CO_2 at 37°C in a fully humidified atmosphere. Exponentially growing cells were used in all experiments.

2.3. Biosynthetic labeling

Cells were starved for 30 min in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum and then subjected to pulse labeling with [^{35}S]methionine (10 μCi/ml) for the times indicated. In chase experiments after pulse labeling, cells were rinsed once in complete medium and incubated for the times indicated.

2.4. Immunoprecipitation

[^{35}S]Methionine-labeled cells were harvested and washed twice in PBS. The cell pellet was suspended in 1 ml of RIPA buffer (30 mM HEPES-NaOH, pH 7.3, containing 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholate and 0.1% SDS) and incubated on ice for 1 h. For protection against proteolytic degradation, a mixture of protease inhibitors (0.2 mM PMSF, 100 μM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml E-64 and 1 μg/ml pepstatin A) was added. The mixture was then centrifuged at 10000×*g* for 10 min at 4°C. 50 μl of protein A-Sepharose CL-4B (1:1 slurry) was added to the resulting supernatant and incubated for 1 h at 4°C. The mixture was centrifuged at 8000×*g* for 5 min at 4°C. Anti-GST-fusion HDC antiserum was added to the resulting supernatant (1:200) and then incubated for 1 h at 4°C. 50 μl of protein A-Sepharose CL-4B was added to the mixture and incubated for 1 h at 4°C. The mixture was recentrifuged at 8000×*g* for 5 min at 4°C, and the resulting precipitate was washed five times with 1 ml of RIPA buffer. The immunoprecipitate was resuspended in an equal volume of 2×SDS sample buffer (125 mM Tris-HCl, pH 6.8, containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.1% bromophenol blue) and boiled for 15 min. The sample was subjected to SDS-PAGE (10% slab gel) according to the method of Laemmli [17]. The gel was dried and analyzed with a Fujix BAS 2000 Bio-Imaging Analyzer.

2.5. Immunoblot analysis

The immunoprecipitate prepared with anti-GST-fusion HDC antiserum was subjected to SDS-PAGE (7% slab gel) and the separated proteins were transferred electrophoretically to a PVDF membrane in 25 mM Tris base containing 40 mM 6-aminohexanoic acid, 0.02% SDS and 20% methanol at room temperature for 90 min at 15 V. The membrane was rinsed in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) and then preincubated overnight in TBS containing 5% non-fat milk at 4°C. The membrane was then incubated with anti-ubiquitin (1:1000) antibody in TBS containing 5% non-fat milk for 1 h at 37°C. The membrane was washed three times with TTBS (TBS containing 0.05% Tween-20) at room temperature. The membrane was incubated with peroxidase-conjugated anti-mouse IgG in TTBS for 1 h at room temperature, and then stained with the ECL Western blot detection reagent.

2.6. In vitro degradation of 74 kDa HDC

The cells labeled with [^{35}S]methionine for 30 min were homogenized, using a Teflon-glass homogenizer, with 40 strokes in the lysis buffer (10 mM HEPES-NaOH, pH 7.3, containing 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA and the mixture of protease inhibitors). The homogenate was centrifuged at 1000×*g* for 10 min at 4°C and then the resultant supernatant was recentrifuged at 10000×*g* for 30 min at 4°C. The supernatant obtained was desalted using a NAP-5 column. The desalted supernatant was incubated for 10 min at 37°C in the (−) ATP buffer (the lysis buffer containing 2 mM ATPγS) and in the (+) ATP buffer (the lysis buffer containing 2 mM ATP, 1 mM creatine phosphate and 37.5 μg/ml creatine phosphokinase). The reaction was stopped by adding 1/5 the volume of 5×RIPA buffer containing a 5×concentration of the mixture of protease inhibitors, and then it was subjected to immunoprecipitation analysis as described above.

3. Results

3.1. Conversion of the [^{35}S]methionine-labeled 74 kDa form into the 53 kDa form of HDC in RBL-2H3 cells

The synthesis of the 74 kDa and 53 kDa forms of HDC was separately analyzed by pulse labeling study (Fig. 1A) and conversion of the former to the latter form by pulse-chase study (Fig. 1B) in RBL-2H3 cells. The radioactive 74 kDa form of HDC was detected within 2 min of pulse labeling and its level increased to a maximum level by 30 min. On the other hand, the radioactive 53 kDa form of HDC appeared 10 min after pulse labeling and its level gradually increased. Furthermore, the pulse-chase study revealed conversion of the radioactive 74 kDa form to its 53 kDa form. The

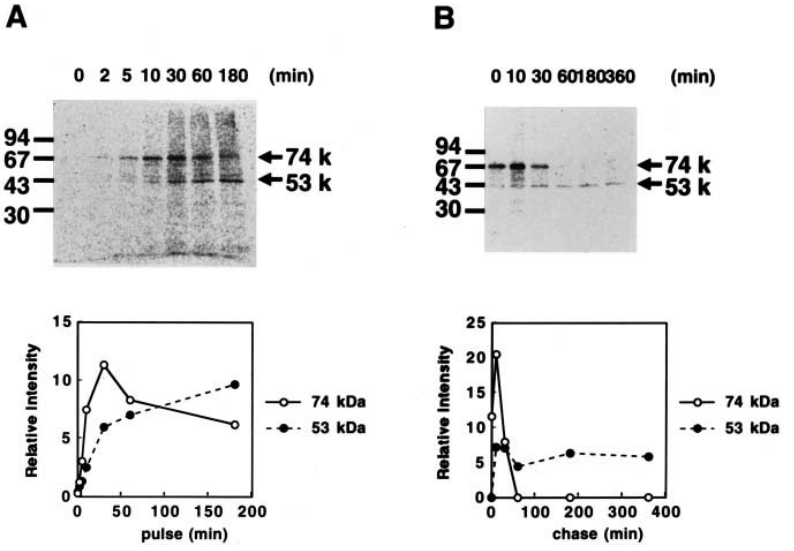

Fig. 1. Time-dependent formation of the 74 kDa and 53 kDa forms of HDC. RBL-2H3 cells (1×10^7 cells/lane) were pulsed with [^{35}S]methionine for the time periods indicated (A), and chased (pulse for 10 min) for the times indicated in the presence of an excess amount of cold methionine (B) before lysis in RIPA buffer and immunoprecipitation with anti-GST-fusion HDC antiserum. The immunoprecipitates were analyzed by SDS-PAGE and with a Fujix BAS 2000 Bio-Imaging Analyzer. The 74 kDa HDC and 53 kDa HDC are indicated by the arrows.

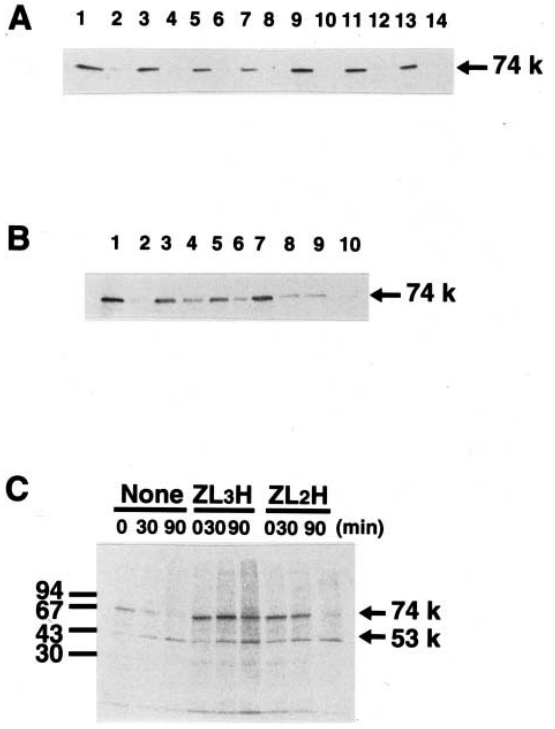

Fig. 2. Effect of protease inhibitors on the degradation of 74 kDa HDC. RBL-2H3 cells (1×10^7 cells) were incubated with or without various protease inhibitors for 3 h, and pulsed with [^{35}S]methionine for 10 min (lanes 1, 3, 5, 7, 9, 11 and 13) followed by a chase for 90 min (lanes 2, 4, 6, 8, 10, 12 and 14). The immunoprecipitated HDCs were analyzed by SDS-PAGE and a Fujix BAS 2000 Bio-Imaging Analyzer. Column A shows the effects of various protease inhibitors; lanes 1 and 2, no treatment; 3 and 4, aprotinin (5 μg/ml); 5 and 6, leupeptin (5 μg/ml); 7 and 8, benzamidine (10 μM); 9 and 10, PMSF (2 μM); 11 and 12, E-64 (5 μg/ml); 13 and 14, pepstatin A (1 μg/ml). Column B shows the effects of proteasome inhibitors; lanes 1 and 2, no treatment; 3 and 4, ZL_3H (20 μM, pretreated for 30 min); 5 and 6, lactacystin (10 μM, pretreated for 3 h); 7 and 8, ALLN (100 μM, pretreated for 30 min); 9 and 10, ZL_2H (20 μM, pretreated for 30 min). Column C shows the effects of ZL_3H and ZL_2H by pulse-chase study. Cells (1×10^7 cells/lane) were pulsed for 10 min and chased for the time periods indicated. 30 min before pulse labeling, 20 μM ZL_3H and 20 μM ZL_2H and dimethylsulfonyloxide (solvent alone) were added to the culture medium. Immunoprecipitated HDCs were analyzed.

amount of the radioactive 74 kDa form synthesized during the 10 min of pulse labeling, sharply reduced to a negligible level over 60 min of pulse chasing. In turn the radioactive 53 kDa form consistently accumulated up to 30 min, and reached a plateau level by 6 h. These profiles of conversion in a 10 min pulse study were basically reproduced in a 30 min pulse study (data not shown).

3.2. *Effect of proteasome inhibitors on the degradation of 74 kDa HDC*

To determine the mechanism of the sharp decrease in the level of the 74 kDa form, we clarified whether the degradation of the radioactive 74 kDa form is sensitive to specific proteinases. For identification of the possible proteinase involved, various proteinase inhibitors were added to cells before treatment with a radioactive pulse for 10 min followed by a chase for 90 min. The radioactive 74 kDa form after the chase was compared for each proteinase inhibitor used. The degradation of the 74 kDa form was largely inhibited in the presence of proteasome inhibitors (Fig. 2B), such as ZL_3H (lane 3 vs. 4), ALLN (lane 5 vs. 6), and lactacystin (lane 7 vs. 8), and slightly by ZL_2H (lane 9 vs. 10) which is a weak inhibitor of proteasome but a potent calpain inhibitor [18]. However, no significant effects were observed using other proteinase inhibitors, such as aprotinin, leupeptin, benzamidine, PMSF, E-64 and pepstatin A (Fig. 2A) or chloroquine, an inhibitor of lysosomal protein degradation (data not shown). As shown in Fig. 2C, the addition of ZL_3H and ZL_2H also increased the accumulation of the radioactive 74 kDa form, but the latter showed short-term activity compared to the former.

3.3. *Ubiquitination of 74 kDa HDC*

In order to investigate the involvement of ubiquitin conjugation in the degradation of the 74 kDa form of HDC, we analyzed the formation of high molecular weight ubiquitin-HDC adductants. The smear bands of polyubiquitinated HDC adductants, having an estimated molecular weight of over 150 kDa, which were immunoprecipitated by anti-GST-fusion HDC antibody and detected with anti-ubiquitin antibody, were marked in the presence of ZL_3H (Fig. 3A). The smear bands comprised radioactive HDC formed during pulse labeling (Fig. 3B).

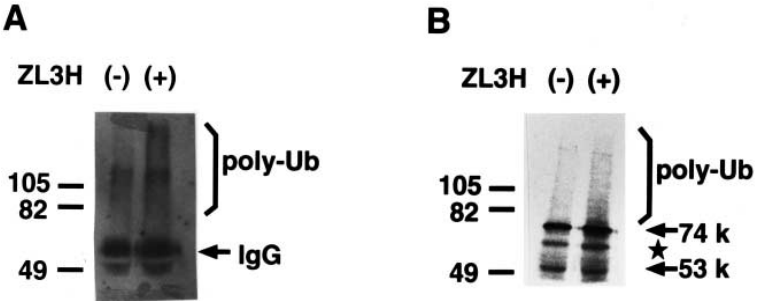

Fig. 3. Ubiquitination of 74 kDa HDC. RBL-2H3 cells (5×10^7 cells) were pretreated with (+) or without (−) 20 μM ZL_3H for 30 min, and pulse-labeled with [^{35}S]methionine for 30 min and immunoprecipitated with anti-GST-fusion HDC antiserum. Immunoprecipitates were subjected to SDS-PAGE (7% slab gel), and separated proteins were transferred to PVDF membrane, and immunoblotted with anti-ubiquitin antibody (1:1000) as described in Section 2. A: Immunoblot analysis with anti-ubiquitin antibody. 'IgG' indicates antibody used in immunoprecipitation (anti-GST-fusion HDC antiserum). B: Autoradiography of the same blot as in A. The blot was analyzed using a Fujix BAS 2000 Bio-Imaging Analyzer. The arrows indicate the 74 kDa and 53 kDa forms of HDC. The star indicates a non-specific band. 'poly-Ub' indicates poly-ubiquitinated 74 kDa HDC.

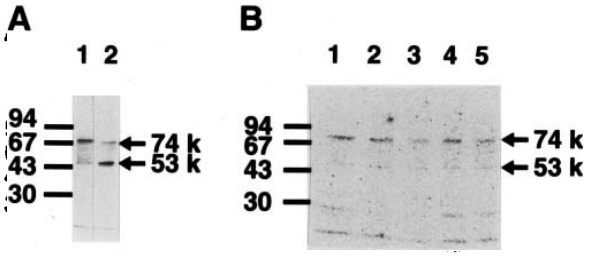

Fig. 4. ATP dependence of the degradation of 74 kDa HDC in vitro. RBL-2H3 cells (1×10^7 cells/lane) labeled with [^{35}S]methionine for 30 min were homogenized and fractionated by centrifugation as described in Section 2. A: Radioactive 74 kDa and 53 kDa HDC in the $10\,000\times g$ supernatant (lane 1) and precipitate fraction (lane 2) after immunoprecipitation with anti-GST-fusion HDC antiserum. B: Changes in radioactive 74 kDa HDC in aliquots of the $10\,000\times g$ supernatant which were incubated for 10 min at 37°C with (lanes 4 and 5) or without (lanes 2 and 3) 20 μM ZL_3H. The supernatant was incubated in the (−) ATP buffer (the lysis buffer containing 2 mM ATPγS) (lanes 2 and 4) and in the (+) ATP buffer (the lysis buffer containing 2 mM ATP, 1 mM creatine phosphate and 37.5 μg/ml creatine phosphokinase) (lanes 3 and 5). Lane 1 is the control without incubation. The arrows indicate the 74 kDa and 53 kDa forms of HDC.

3.4. ATP dependence of the degradation of 74 kDa HDC in vitro

To determine whether the degradation of the 74 kDa form of HDC is energy-dependent, the radioactive 74 kDa form in the supernatant fraction obtained by centrifugation at $10\,000\times g$ for 60 min was treated with or without an ATP-generating system in the presence or absence of ZL_3H in vitro. The $10\,000\times g$ supernatant fraction of the cells contained the majority of the 74 kDa form (Fig. 4A, lane 1), and the $10\,000\times g$ precipitate contained the 53 kDa form. During the incubation for 10 min at 37°C, the level of the radioactive 74 kDa form was not changed substantially in the presence of ATPγS, but was apparently decreased in the presence of the ATP-generating system (Fig. 4B, lane 2 vs. 3). This ATP-dependent degradation of the 74 kDa form was partially reduced by addition of ZL_3H (lane 3 vs. 5).

3.5. Proteasome activity in the extract of RBL-2H3 cells

To confirm that RBL-2H3 cells contain proteasome activity, we assayed the hydrolysis of z-leu-leu-leu-MCA, a preferential substrate of the proteasome [19]. The $10\,000\times g$ supernatant of the cells exhibits a significant amount of hydrolytic activity (148 ± 24.0 nmol/min/10^7 cells) which was completely abolished in the presence of 20 μM ZL_3H (0 ± 0 nmol/min/10^7 cells).

4. Discussion

Recent studies have suggested that the proteasome pathway is involved in the selective degradation of many important proteins such as muscle proteins, cell cycle regulatory proteins, transcription factors, and enzymes such as tyrosine aminotransferase and ODC [10,11]. Proteasome catalyzes an energy-dependent, non-lysosomal proteolytic pathway. Here we demonstrated that the degradation of the 74 kDa form of HDC in RBL-2H3 cells was ATP-dependent, under the ubiquitin-conjugated pathway, and sensitive to proteasome inhibitors. These results, together with the significant activity of proteasome in RBL-2H3 cells, demonstrated that the 74 kDa form is a short half-life protein because of its rapid degradation via the proteasome pathway. Compared to the 74 kDa form, the 53 kDa form seems to be relatively stable (Fig. 1B). As shown in Fig. 4, the 74 kDa form is localized primarily in the cytosol and partially in the particulate fraction, whereas the 53 kDa form is mostly located in the particulate fraction, which has been revealed to consist of the endoplasmic reticulum (ER), Golgi and granules (data not shown). In contrast to the present study, we previously reported that the expressed recombinant 74 kDa form of HDC in Sf9 cells was mostly recovered in the particulate fraction [8]. Considering the rapid degradation of the 74 kDa form, it is possible that the precipitated recombinant 74 kDa form of HDC in Sf9 cells is an abnormal form of protein similar to bacterial inclusion bodies, which have been reported in a bacterial system. Although the soluble recombinant 54 kDa form of HDC was observed in Sf9 cells [8], this form may not reflect the nature of the 53 kDa form generated through post-translational processing of 74 kDa HDC in RBL-2H3 cells.

Since the 74 kDa form lacks an amino-terminal signal sequence that is cleaved following translocation across the ER membrane [5], it may be synthesized by free ribosomes in the cytosol as a non-secreted protein. The existence of the particulate 53 kDa form means the targeting and conversion of the 74 kDa form in the particulate fraction. As a result, the 74 kDa form of HDC is subjected to metabolism via two distinct ways in the cytosol: targeting to the ER resulting in the formation of the 53 kDa form and degradation via the ubiquitin-proteasome pathway. A rapid decrease in the concentration of the radioactive 74 kDa form was not accompanied by an increase in the concentration of the 53 kDa form and the amount of 53 kDa form did not change during a chase period (Fig. 1B). In addition, the pretreatment of proteasome inhibitors did not change the amount of the 53 kDa form (Fig. 2C). These results indicate that ubiquitin-proteasome pathway may not be involved in the conversion of the 74 kDa form into the 53 kDa form.

ODC, like HDC, is a very unstable protein [20] and is known to be degraded by the ATP/antizyme-dependent proteasome pathway [21]. Recently, some common structural features of ODC and HDC were identified [22]. These include the presence of PEST regions. PEST regions can act as constitutive or conditional signals providing a means for the intracellular degradation of key metabolic proteins [23]. Rat HDC has three PEST regions in its amino acid sequence [22]: one between amino acids 43–74 which is homologous to the PEST region in mouse and human HDC and two between amino acids 503–525 and 529–555 which are present in the C-terminal region of the 74 kDa form but not in the 53 kDa form of HDC. The two PEST regions in the C-terminal region of the 74 kDa form have a role in the degradation via the proteasome pathway. The degradation of ODC is not dependent on ubiquitination [15], which is responsible for the degradation of HDC. Here we first demonstrated that HDC is degraded by proteolysis mediated by the ATP/ubiquitin-dependent proteasome pathway.

At present the reason for the existence of the two forms of HDC, the soluble 74 kDa form and the particulate 53 kDa form, in RBL-2H3 cells is unclear. The 74 kDa form may have a role in the production of cytosolic histamine which is spontaneously released or transported to histamine-containing

granules for storage, whereas the particulate 53 kDa form is convenient to directly synthesize granular histamine which is released in response to a receptor-mediated immune reaction. The function of cytosolic histamine remains to be clarified, but it is possible that it is involved in the growth of some cell lines and tumors [24–26]. To test this hypothesis, it will be necessary to determine in detail the localization of two forms of HDC and the characteristics of the converting enzyme involved in the synthesis of the 53 kDa form from the 74 kDa form of HDC.

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