*Articles*

# Quantity and Quality Control of Gastric Proton Pump in the Endoplasmic Reticulum by Ubiquitin/Proteasome System†

Tohru Kimura,‡ Hokara Ishizuka,‡ Ayumi Yoshida,‡ Magotoshi Morii,‡ Noriaki Takeguchi,‡ and Shinji Asano*,#

*Faculty of Pharmaceutical Sciences and Life Science Research Center of Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan*

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ABSTRACT: The gastric proton pump, $H^+$,$K^+$-ATPase, consists of the catalytic α-subunit and the noncatalytic *β*-subunit. These subunits are assembled in the endoplasmic reticulum (ER) and leave the ER to reach to the cell surface as a functional holoenzyme. We studied the quantity control mechanism of the $H^+$,$K^+$-ATPase in the ER by using a heterologous expression system in human embryonic kidney 293 cells. The α-subunit in the α-expressing cells was degraded more rapidly than in the α+*β*-expressing cells. It was stabilized, however, in the presence of a proteasome inhibitor, lactacystin. Polyubiquitination of the α-subunit was observed in the α-expressing cells as well as in the α+*β*-expressing cells. The extent of polyubiquitination was higher in the former α-expressing cells especially in the presence of lactacystin. On the other hand, polyubiquitination of the *β*-subunit was not observed in the absence and presence of lactacystin. When the α-subunit was coexpressed with a mutant *β*-subunit that lacks α/*β* assembly capacity, degradation of the α-subunit was accelerated in parallel with increased polyubiquitination of the α-subunit. These results indicate that the ubiquitin/proteasome system is involved in degradation of the unassembled α-subunits in the ER to control the cell surface expression of the functional α/*β* holoenzymes.

The gastric proton pump, $H^+$,$K^+$-ATPase, actively transports proton and $K^+$ in the opposite directions, coupled with the hydrolysis of ATP. This pump consists of the catalytic α- and the noncatalytic *β*-subunits. The α-subunit, with a molecular mass of 114 kDa, contains sites for ATP-binding (*1*, *2*) and its acyl-phosphorylation (*3*), sites for binding of proton pump inhibitors (*4*−*7*), and sites responsible for ion recognition (*6*, *8*−*11*). The *β*-subunit, with a molecular mass of 33 kDa (as a protein core), is also essential for the functional expression of $H^+$,$K^+$-ATPase through interactions with the α-subunit (*8*, *12*−*14*). The interaction between the α- and *β*-subunits seems to be important for the conformational stability of the functional holoenzyme (*15*, *16*). The *β*-subunit especially supports the membrane insertion of the carboxy-terminal part (M5 to M10 transmembrane segments) of the α-subunit, resulting in the stabilization of the α-subunit and α/*β* holoenzyme (*17*).

The *β*-subunit is also important for intracellular trafficking of the α- and *β*-subunits. Only the α-subunit that is assembled with the *β*-subunit leaves the endoplasmic reticulum (ER)[1] to travel to the cell surface, whereas the α-subunit free of the *β*-subunit is specifically retained and degraded in the ER both in the stable and transient expression systems (*16*, *18*). On the other hand, the *β*-subunit can leave the ER without the α-subunit.

The genes encoding the α- and *β*-subunits are located at different chromosomal loci (chromosomes 19q13.11 and 13q34, for human α- and *β*-subunits, respectively) (*19*, *20*). These two subunits are cotranslationally inserted into the ER membrane, and assembled into the functional holoenzyme with the stoichiometry of 1 to 1. Therefore, strict control mechanisms seem to regulate the quantity (number) of these subunits in the ER. However, little is known about the mechanism for the quality and quantity control of gastric $H^+$,$K^+$-ATPase.

Intracellular degradation of proteins in mammalian cells is due to the combined action of the proteasome, a cytosolic multisubunit protease of broad specificity, and the lysosome, an acidic compartment of cells in which numerous proteases and glycosidases are localized. The proteasome is a protease complex (26S proteasome) comprising the catalytic core (20S

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* To whom correspondence should be addressed.Tel: +81−76−434−7187. Fax: +81−76−434−5176. E-mail: shinji@ms.toyama-mpu.ac.jp.

‡ Faculty of Pharmaceutical Sciences.

# Life Science Research Center.

[1] Abbreviations: ER, endoplasmic reticulum; CFTR, cystic fibrosis transmembrane conductance regulator, ENaC, epithelial $Na^+$ channel.

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complex) and the 19S "Cap" structure that stimulates the 20S complex (*21*, *22*). The covalent attachment of chains of ubiquitin (with a molecular mass of 8.5 kDa) to target proteins serves as a signal for their degradation by proteasomes (*23*). Originally, the proteasome was thought to be involved in degradation of soluble and secretary proteins, especially short-lived proteins such as cyclins responsible for cell cycle control (*24*). The proteasome is also involved in limited processing of NF-$\kappa$B precursor (p105) to the 50-kDa active form (p50) (*25*), and degradation of oncoproteins such as c-Jun and tumor suppressor gene product, p53 (*26*, *27*). However, recent findings indicated that the proteasome is also involved in degradation of misfolded or immature membrane proteins retained in the ER (*28*). For example, cystic fibrosis transmembrane conductance regulator (CFTR), both wild type and mutant $\Delta$F508 (which is a major form found in cystic fibrosis patients (*29*)), amiloride-sensitive epithelial $Na^+$ channel (ENaC) (*30*), $Na^+$,$K^+$-ATPase $\alpha$-subunit (*31*, *32*), and aquaporin-1 (*33*) were reported to be polyubiquitinated, and degraded by the proteasome. Here, we studied the role of the ubuquitin/proteasome system on the quality and quantity control of gastric $H^+$,$K^+$-ATPase.

## EXPERIMENTAL PROCEDURES

*Materials.* HEK-293 cells (human embryonic kidney cell line) were a kind gift from Prof. Jonathan Lytton (University of Calgary, Calgary, Canada). The pcDNA3.1/ZEO(+) and pcDNA3 vectors were obtained from Invitrogen Co. (San Diego, CA). Effectene Transfection Reagent and EndoFree Plasmid Maxi and Mega kits were obtained form QIAGEN (Tokyo, Japan). Restriction enzymes and other DNA and RNA modifying enzymes were from Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), or Amersham Pharmacia Biotech. Inc. (Tokyo, Japan). Anti-gastric $H^+$,$K^+$-ATPase $\alpha$-subunit monoclonal antibody, 1H9 and anti-$\beta$-subunit monoclonal antibody, 2B6 were obtained from Molecular Biological Laboratories (Nagoya, Japan). Anti-gastric $H^+$,$K^+$-ATPase $\alpha$-subunit polyclonal antibody HK9 was a kind gift from Prof. Michael Caplan (Yale University, New Haven, CT). Anti-multi-ubiquitin antibody was obtained from Sigma-Aldrich (St. Louis, MO). Lactacystin and epoxomicin were from Peptide Research Lab. (Suita, Japan). All other reagents were of molecular biology grade or the highest grade of purity available.

*Transfection and Selection of Stable Cell Lines.* Cell culture of the HEK-293 cell line was carried out as described previously (*6*, *8*). Stable cell lines expressing the gastric $H^+$,$K^+$-ATPase subunit(s) were constructed as described previously (*16*, *34*). For construction of the $\beta$-expressing cell line, HEK-293 cells were transfected with the pcDNA3-HK$\beta$ cDNA construct by lipofection using an Effectene Transfection Reagent, and stable cell lines were selected in the presence of 1 mg/mL Geneticin (G-418 sulafate). Single colonies were isolated, expanded, and maintained in the presence of 0.5 mg/mL Geneticin. For construction of the $\alpha$-expressing cell line, HEK-293 cells were transfected with the pcDNA3.1/ZEO(+)-HK$\alpha$ cDNA construct, and stable cell lines were selected in the presence of 0.1 mg/mL Zeocin. Single colonies were isolated, expanded, and maintained in the presence of 0.1 mg/mL Zeocin. Cloning was performed by limited dilution in 96-well microplates. The expression of the $\alpha$- or $\beta$-subunit was confirmed by immunofluorescence and Western blot. For construction of the $\alpha+\beta$-expressing cells, the $\beta$-expressing cell line was transfected with the pcDNA3.1/ZEO(+)-HK$\alpha$ cDNA construct, and stable cell lines were selected in the presence of 0.2 mg/mL Zeocin plus 0.5 mg/mL Geneticin. Single colonies were isolated, expanded, and maintained in the presence of 0.5 mg/mL Geneticin and 0.2 mg/mL Zeocin. The expression of the $\alpha$- and $\beta$-subunits was confirmed by immunofluorescence and Western blot.

*Construction of Mutant $\beta$-Subunit, L-1,2,3, in which All the Disulfide Bonds Were Disrupted by Mutation.* Mutant $\beta$-subunit (L-1,2,3) was constructed by replacing all of six extracellular cysteine residues by serine residues as described previously (*34*).

*Preparation of Membrane Fractions.* Membrane fractions from stable cell lines were prepared as described previously (*8*). Briefly, cells in a 10 cm Petri dish were washed with PBS, and incubated in 2 mL of low ionic salt buffer (0.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) at 0 °C for 10 min. After addition of phenylmethylsulfonyl fluoride (1 mM) and aprotinin (0.09 units/mL), the cells were homogenized in a Dounce homogenizer, and the homogenate was diluted with an equal volume of a solution composed of 500 mM sucrose and 10 mM Tris-HCl, pH 7.4. The homogenized suspension was centrifuged at 800*g* for 10 min. The supernatant was centrifuged at 100000*g* for 90 min, and the pellet was suspended in a solution composed of 250 mM sucrose and 5 mM Tris-HCl, pH 7.4.

*SDS−Polyacrylamide Gel Electrophoresis and Western Blotting.* SDS−polyacrylamide gel electrophoresis was carried out as described elsewhere (*35*). Membrane preparations (30 $\mu$g of protein) were incubated in a sample buffer composed of 2% SDS, 2% $\beta$-mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 6.8 at room temperature for 2 min and separated on an SDS−polyacrylamide gel. Western blotting was carried out as described previously (*8*).

*Antibodies.* Anti-$\alpha$-subunit antibody, Ab1024 was previously raised against the carboxy terminal peptide (Residues 1024−1034) of the $H^+$,$K^+$-ATPase $\alpha$-subunit (*36*). Anti-$\alpha$-subunit monoclonal antibody, 1H9, and anti-$\beta$-subunit monoclonal antibody, 2B6, were derived from the splenocytes of mice with autoimmune gastritis (*37*).

*Immunohistochemistry.* Stable cell lines were fixed for 10 min in cold methanol (−20 °C) and washed three times with PBS. Cells were permeabilized in a permeabilization buffer composed of 0.3% Triton X-100 and 0.1% BSA in PBS for 15 min at room temperature. Nonspecific antibody binding was blocked by preincubation of cells in a goat serum dilution buffer solution (16% goat serum, 0.3% Triton X-100, 0.9% NaCl, and 20 mM sodium phosphate, pH 7.4) for 30 min. All antibody incubations and all washes between antibody incubations were carried out using the goat serum dilution buffer solution. Cells were incubated for 1 h at room temperature with anti-$\alpha$-subunit (HK9) or anti-$\beta$-subunit (2B6) antibody, followed by three washes with the permeabilization buffer. Rhodamine-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG secondary antibodies were used for 1 h incubation at room temperature at a 1:100 dilution. Immunofluorescence images were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

*Glycosidase Treatment.* Membrane fractions were treated with 1 unit of *N*-glycosidase F in a solution composed of

0.1% SDS, 1% *n*-octylglucoside, 1% 2-mercaptoehanol, 30 mM EDTA, and 50 mM sodium phosphate, pH 6.0 at 37 °C overnight as described previously (*38*).

*Pulse-Chase Labeling and Immunoprecipitation.* Pulse-chase labeling of stable cell lines was performed as described previously (*34*). Stable cell lines were cultured on collagen coated 6-well plates. Cells were washed and incubated at 37 °C for 30 min in methionine-free medium. Cells were labeled for 60 min with [$^{35}$S]-Met, Cys Labeling mixture (Express) (NEN), and chased in complete Dulbecco's modified Eagle medium for indicated periods. Cells were washed with a washing buffer composed of 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, and incubated in 500 $\mu$L of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, at 4 °C for 30 min. After centrifugation of the sample at 16000*g* for 20 min, the supernatant was incubated with anti-α-subunit antibody 1H9 or anti-$\beta$-subunit antibody 2B6 at a 1:100 dilution, and 10 $\mu$L of ImmunoPure Immobilized Protein A (Pierce) at 4 °C for 12 h. After centrifugation of the sample, the pellet was washed four times with the lysis buffer followed by two washes with 0.1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The pellet was solubilized in the sample buffer for SDS−polyacrylamide gel electrophoresis, and incubated at room temperature for 10 min. The proteins were separated on an SDS−polyacrylamide gel and were visualized by digital autoradiography of dried gels using Bio Imaging analyzer BAS 2000 (Fuji Photo Film, Tokyo).

*Detection of Polyubiquitination of the* α*- and* $\beta$*-Subunits.* Stable cell lines were incubated in 1 mL of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, at 4 °C for 30 min. The solubilized fraction was incubated with anti-α-subunit antibody HK9 or anti-$\beta$-subunit antibody 2B6, and ImmunoPure Immobilized Protein A at 4 °C for 12 h. After centrifugation of the sample, the pellet was washed four times with the lysis buffer followed by two washes in 0.1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4. The pellet was solubilized in the sample buffer for SDS−polyacrylamide gel electrophoresis, and incubated at room temperature for 10 min. Following separation by SDS−PAGE, proteins were transferred electrophoretically to nitrocellulose paper. Polyubiquitination of the α-subunit in the blots was detected by mouse anti-polyubiquitin antibody in combination with a peroxidase-conjugated anti-mouse antibody, which was preabsorbed with rabbit serum.

Polyubiquitination of the $\beta$-subunit in the blots was detected by rabbit anti-ubiquitin antibody in combination with a peroxidase-conjugated anti-rabbit antibody, which was preabsorbed with mouse serum. Polyubiquitination of the α-subunit was also studied by Western blotting of the anti-polyubiquitin immunoprecipitates detected by the anti-α-antibody.

## RESULTS

*Expression of the* α*- and* $\beta$*-Subunits in Stable Cell Lines.* The expression and localization of the α- and $\beta$-subunits in stable cell lines (the α-expressing cells, the $\beta$-expressing cells, and the α+$\beta$-expressing cells) were studied by immunofluorescence techniques using a confocal laser scanning microscope (Figure 1). In the α-expressing cells, the α-subunit was observed only in the intracellular compartment, and the cell surface expression was not observed (Figure 1A). In the $\beta$-expressing cells, the $\beta$-subunit was observed at the cell surface as well as in the intracellular compartment (Figure 1B). In the α+$\beta$-expressing cells, both subunits were mainly observed at the cell surface (Figure 1C,D). These results indicate that the $\beta$-subunit by itself can leave the ER to travel to the cell surface, whereas the α-subunit cannot travel to the cell surface without the $\beta$-subunit as previously reported (*34*).

Figure 2A shows the Western blots of the α-subunit in the cell lysate prepared from the α-expressing and α+$\beta$-expressing cells. The expression of the α-subunit as a 100-kDa band in the α-expressing cells was significantly lower than that in the α+$\beta$-expressing cells, as was reported previously based on studies employing a transient expression system (*8*). To study the role of protein degradation by the proteasome system in the steady-state expression level of the α-subunit, we cultured the cells in the presence of lactacystin, and performed Western blotting of the cell lysate with an anti-α-subunit antibody. Lactacystin specifically inhibits peptidase activities of the proteasome by covalently modifying the amino-terminal threonine residue on the mammalian proteasome $\beta$-subunits (*39*). In the presence of lactacystin, the α-subunit was observed as a smeary pattern with a molecular mass higher than 100 kDa. This result suggests that the α-subunit was modified with chains of polyubiquitin. Polyubiquitination of the α-subunit was studied by immunoprecipitation, which is presented later in the present paper. The expression level of the α-subunit in the α-expressing cells was significantly increased in the presence of lactacystin.

Figure 2B shows the Western blots of the $\beta$-subunit in the cell lysate prepared from the $\beta$-expressing and α+$\beta$-expressing cells. A dense smeary band with a molecular mass of 60−70 kDa ($\beta_m$) and a band with a molecular mass of 48 kDa ($\beta_c$) were observed in each lane. The $\beta_m$ and $\beta_c$ represent the $\beta$-subunit with complex-type carbohydrate chains (mature glycosylation) and that with high mannose-type carbohydrate chains (core glycosylation), respectively, judged by the sensitivity to endoglycosidase H (*38*). The expression level of the $\beta$-subunit in the α+$\beta$-expressing cells was similar or slightly lower than that in the parental $\beta$-expressing cells. Lactacystin did not significantly increase the expression levels of the $\beta$-subunit in both the $\beta$-expressing and α+$\beta$-expressing cells.

*Pulse-Chase Labeling of the* α*-Expressing,* $\beta$*-Expressing, and* α+$\beta$*-Expressing Cells Followed by Immunoprecipitation with the Anti-*α*- or Anti-*$\beta$*-Subunit Antibody.* To precisely study the stability of the α- and $\beta$-subunits, pulse-chase labeling experiments of the α-expressing, $\beta$-expressing, and α+$\beta$-expressing cells were performed. Cells were labeled with [$^{35}$S]Met/Cys for 1 h, followed by cold chase for various periods, and their cell lysate samples were immunoprecipitated with the anti-α- or anti-$\beta$-subunit antibody, respectively. The precipitated proteins were separated on SDS−polyacrylamide gels, and visualized by digital autoradiography (Figures 3 and 4).

Expression of the α-subunit in the α-expressing cells in a 1 h pulse period (chase time, 0) in the absence of lactacystin was much higher than that in the α+$\beta$-expressing cells. The

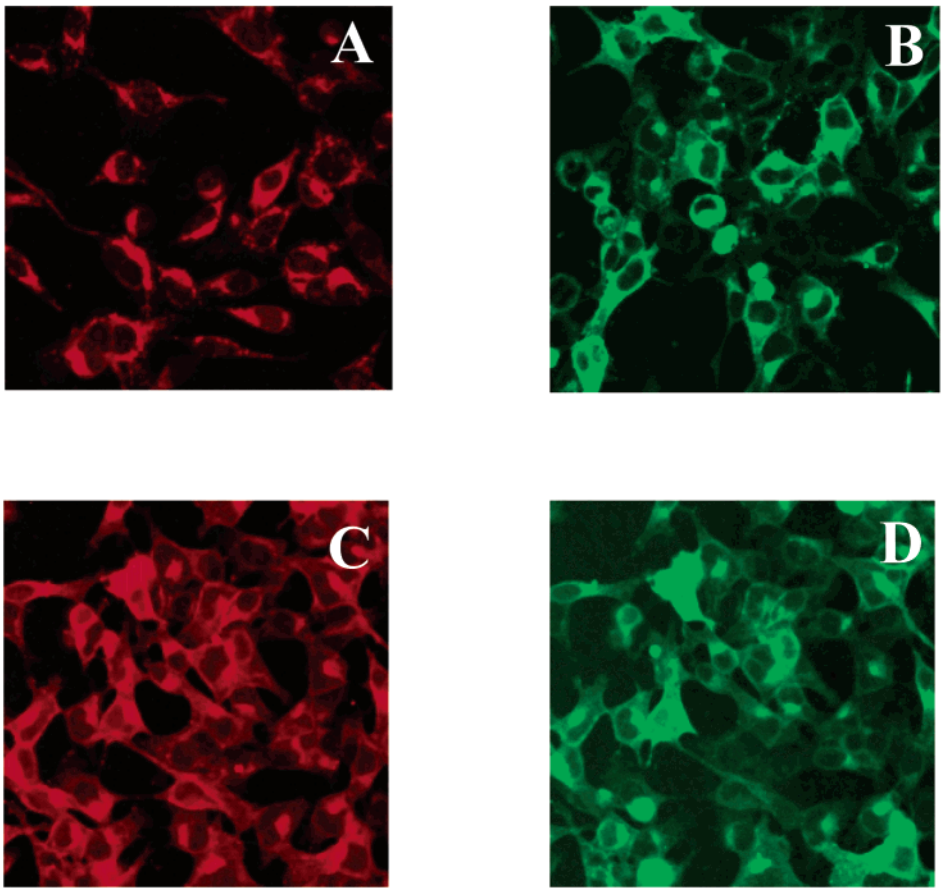


FIGURE 1: Immunolocalization of the α- and *β*-subunits expressed in stable cell lines. The α-expressing cells (A) or the *β*-expressing cells (B) were stained with an anti-α-antibody, HK9 or an anti-*β*-antibody, 2B6, respectively. The α+*β*-expressing cells were stained with HK9 (C) and 2B6 antibodies (D), respectively.

*β*-subunit seems to negatively regulate the translation of the α-subunit through co-translational assembly with the α-subunit. In the α-expressing cells, degradation of the α-subunit was apparent within 1 h, and was almost complete within 6 h. However, the α-subunit was stable for much longer time in the α+*β*-expressing cells, and more than 70% of the α-subunit still remained without degradation after 6 h pulse period (Figure 3C).

To study the role of protein degradation by the proteasome systems in the stability of the α-subunit, we performed pulse-chase labeling experiments in the presence of lactacystin. The α-subunit in the α-expressing cells was significantly stabilized by lactacystin. Lactacystin prevented the degradation of the α-subunit in the α-expressing cells for at least 3 h of the chase period (Figure 3A,C). This result suggests that unassembled α-subunits retained in the ER were specifically degraded by proteasomes. The α-subunit was rapidly degraded between 3 and 6 h chase period even in the presence of this inhibitor. The α-subunit may be degraded by proteases other than proteasomes in the presence of lactacystin.

The *β*-subunit in the *β*-expressing and α+*β*-expressing cells was modified with complex-type carbohydrate chains ($\beta_m$) within 3 h chase period (Figure 4). The $\beta_m$ was stable at least between 3 and 6 h chase period. The process of modification of the carbohydrate chains of the *β*-subunit was almost unchanged between the *β*-expressing cells and the α+*β*-expressing cells (Figure 4). Lactacystin did not affect the stability of the $\beta_c$ in both the *β*-expressing and α+*β*-expressing cells. Intracellular trafficking of the *β*-subunit from the ER to the trans-Golgi was judged not to be inhibited by lactacystin both in the *β*-expressing and α+*β*-expressing cells because modification of the carbohydrate chains of the *β*-subunits from high mannose-type ($\beta_c$) to complex-type ($\beta_m$) proceeded even in the presence of this inhibitor. However, it should be pointed out that lactacystin stabilized the $\beta_m$ in the *β*-expressing cells and α+*β*-expressing cells. The $\beta_m$ was observed as a dense band after 6 h chase period in the presence of lactacystin. These results may suggest that the *β*-subunits traveling to the trans-Golgi or the cell surface are degraded by proteasomes. Similar results were observed in the pulse-chase experiment in the presence of epoxomicin, a different inhibitor specific for proteasome (data not shown) (*40*).

*Polyubiquitination of the* α- *and* *β*-*Subunits*. Proteasomes recognize chains of ubiquitin polymer covalently attached to target proteins as a signal for their degradation. Ubiquitin is a polypeptide with a molecular mass of 8.5 kDa consisting of 76 amino acids, and is linked to target proteins through the formation of an isopeptide bond between its carboxyl terminus and the *ϵ*-amino group of lysine residue of target proteins (*41*). We studied whether the α- and *β*-subunits were polyubiquitinated in each cell line.

To confirm polyubiquitination of the α-subunit, cell lysate samples prepared from the α-expressing and α+*β*-expressing cells cultured in the presence or absence of lactacystin were immunoprecipitated with the anti-α-subunit antibody, followed by Western blotting with an anti-polyubiquitin anti-

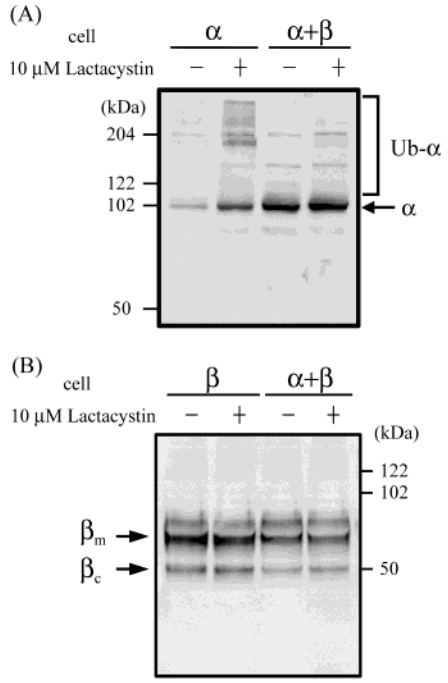


FIGURE 2: Western blots with an anti-α-subunit (A) or an anti-*β*-subunit antibody (B) of the cell lysate preparations from the α-expressing and α+*β*-expressing cells (A), and the *β*-expressing and α+*β*-expressing cells (B) cultured in the presence or absence of 10 *μ*M lactacystin for 3 h. Each stable cell line was solubilized in 1 mL of lysis buffer composed of 1% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.4, at 4 °C for 30 min. The cell lysate (30 *μ*g) was separated on an SDS−polyacrylamide gel and subjected to Western blotting with anti-α-subunit antibody Ab1024 (A) or anti-*β*-subunit antibody 2B6 (B). α- and Ub-α shown on the right side (A) represent the unubiquitinated and ubiquitinated α-subunits, respectively. $\beta_m$ and $\beta_c$ shown on the left side (B) represent the *β*-subunits modified with complex-type (mature glycosylation) and high mannose-type carbohydrate chains (core glycosylation), respectively.

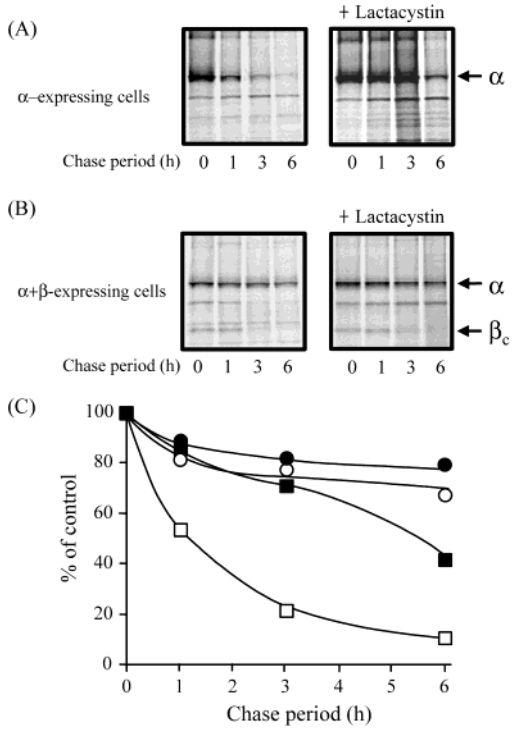


FIGURE 3: Pulse chase labeling of the α-expressing cells (A) and the α+*β*-expressing cells (B), followed by the immunoprecipitation with an anti-α-subunit antibody. Stable cell lines cultured on collagen-coated six-well plates were labeled for 60 min with [$^{35}$S]-Met, Cys Labeling mixture (EXPRESS), followed by a chase in the complete medium for indicated periods in the presence and absence of 10 *μ*M lactacystin. Cells were solubilized in 500 *μ*L of lysis buffer at 4 °C for 30 min. After centrifugation, the supernatant was incubated with anti-α-subunit antibody 1H9, and ImmunoPure Immobilized Protein A at 4 °C for 12 h. After centrifugation, the pellet was solubilized in the sample buffer for SDS−polyacrylamide gel electrophoresis, separated on the gel, and visualized by digital autoradiography. α and $\beta_c$ represent the α-subunit and the *β*-subunit modified with high mannose-type carbohydrate chains, respectively. (C) Quantification of the intensity of the bands representing the α-subunit shown in panels A and B. The quantity of the α-subunit after each chase period was counted from the density of the digital autoradiography, and expressed as the percentage of that at 0 time. □ and ■ represent the quantity of the α-subunit in the α-expressing cells in the absence and presence of lactacystin, respectively. ○ and ● represent the quantity of the α-subunit in the α+*β*-expressing cells in the absence and presence of lactacystin, respectively.

body. Polyubiquitination of the α-subunit was observed as a smeary band with a molecular mass of 100−300 kDa, especially in the membrane fraction prepared from the α-expressing cells, and was enhanced when the cells were cultured in the presence of lactacystin (Figure 5A). The density of the band was high in a region of higher than 200 kDa of molecular mass. A similar pattern was found in the Western blots of the anti-polyubiquitin antibody immunoprecipitates detected with an anti-α-subunit antibody (Figure 5B). Bands with a molecular mass of 100 kDa found in Figure 5B are nonspecific because this band was observed in mock-transfected cells. This result indicates that the smeary band with a molecular mass of 100−300 kDa does not represent ubiquitinated proteins co-immunoprecipitated with the α-subunit. A similar pattern was also observed in the Western blots of the anti-α-subunit immunoprecipitates from cell lysate samples detected with the anti-α-subunit antibody (Figure 5C). A lower level of polyubiquitination of the α-subunit was also observed in the α+*β*-expressing cells cultured in the presence of lactacystin (Figure 5A).

Next, cell lysate samples prepared from the *β*-expressing and α+*β*-expressing cells were immunoprecipitated with an anti-*β*-subunit antibody, followed by Western blotting with an anti-multi-ubiquitin antibody. Polyubiquitination of the *β*-subunit was not observed when the cells were cultured either in the presence or absence of lactacystin (data not shown). This result was further supported by the finding that after complete removal of carbohydrate chains by the treatment of *N*-glycosidase F, the *β*-subunit was observed as a clear single band with a molecular mass of 33 kDa in the Western blot of membrane fractions prepared from the *β*-expressing and α+*β*-expressing cells (data not shown). These results indicate that the *β*-subunit is not polyubiquitinated either in the presence or absence of lactacystin.

These results presented above strongly suggest that unassembled α-subunits are specifically retained in the ER, modified with chains of ubiquitin polymer, and degraded by proteasomes.

*Pulse-Chase Labeling of the α-Subunit and Mutant β-Subunit that Lacks Disulfide Bonds.* To study whether similar phenomena occur in the α+*β*-expressing cells when correct assembly between the α- and *β*-subunits is prevented, we performed pulse-chase labeling of the stable cell line coexpressing the wild-type α-subunit plus mutant *β*-subunit (L-1,2,3), in which no disulfide bond was formed because

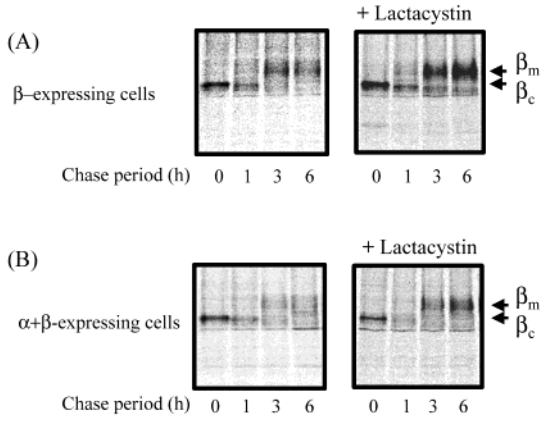


FIGURE 4: Pulse chase labeling of the $\beta$-expressing cells (A) and the $\alpha+\beta$-expressing cell (B), followed by the immunoprecipitation with an anti-$\beta$-subunit antibody. Stable cell lines cultured on collagen-coated six-well plates were labeled for 60 min with [$^{35}$S]-Met, Cys Labeling mixture (EXPRESS), followed by a chase in the complete medium for indicated periods in the presence and absence of 10 $\mu$M lactacystin. Cells were solubilized in 500 $\mu$L of lysis buffer at 4 °C for 30 min. After centrifugation of the sample, the supernatant was incubated with anti-$\beta$-subunit antibody 2B6, and ImmunoPure Immobilized Protein A at 4 °C for 12 h. After centrifugation of the sample, the pellet was incubated in the sample buffer for SDS−polyacrylamide gel electrophoresis, separated on a gel, and visualized by digital autoradiography. $\beta_m$ and $\beta_c$ represent the $\beta$-subunits modified with complex-type and high mannose-type carbohydrate chains, respectively.

all of six extracellular cysteine residues of the $\beta$-subunit had been replaced by serine residues (Figure 6). Previously, we found that the L-1,2,3 mutant $\beta$-subunit did not assemble with the α-subunit and that when mutant L-1,2,3 was coexpressed with the α-subunit, both the α-subunit and this mutant $\beta$-subunit were retained in the ER, and the $H^+,K^+$-ATPase activity was not observed (*34*). Here, the α-subunit coexpressed with mutant L-1,2,3 $\beta$-subunit was found to be unstable in the absence of lactacystin; degradation of the α-subunit was almost complete in 6 h chase period (Figure 6). However, the α-subunit was significantly stabilized by the incubation with lactacystin; 75 and 50% of the α-subunits were retained after 3 and 6 h chase period, respectively (Figure 6C).

Mutant L-1,2,3 $\beta$-subunit was not modified with complex-type carbohydrate chains in the presence and absence of lactacystin, indicating that the L-1,2,3 mutant was retained in the ER. The L-1,2,3 mutant was stabilized in the presence of lactacystin, similar to the α-subunit as shown in Figure 7.

*Expression of the* α*-Subunit in a Stable Cell Line Co-expressing the Wild-type* α*-Subunit and the Mutant* $\beta$*-Subunit that Lacks Disulfide Bonds*. Figure 8 shows the Western blots with an anti-α-subunit antibody of the cell lysate samples prepared from the α-expressing, $\alpha+\beta$-expressing, and α+L-1,2,3-expressing cells in the presence and absence of lactacystin. In the absence of lactacystin, the expression level of the α-subunit in the α+L-1,2,3-expressing cells was significantly lower than that in the $\alpha+\beta$-expressing cells. The presence of lactacystin increased the expression level of the α-subunit in the α+L-1,2,3-expressing cells, and the ubiquitinated α-subunit was observed as a smeary pattern with a molecular mass higher than 100 kDa. These results are comparable with those found in the α-expressing cells,

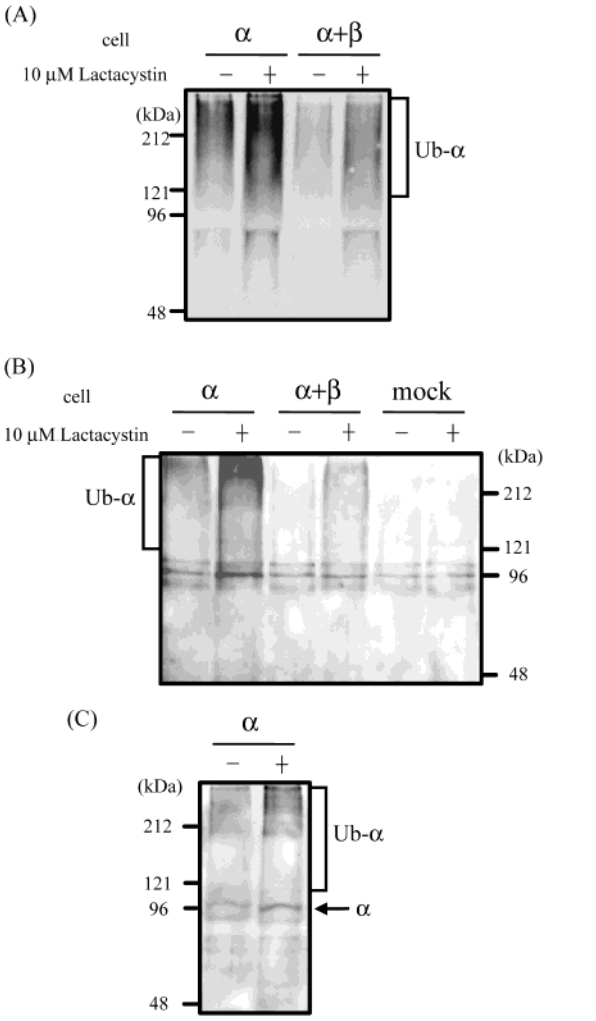


FIGURE 5: (A) Western blots with an anti-polyubiquitin antibody of the anti-α-subunit immunoprecipitates from lysate of stable cell lines. The α-expressing and $\alpha+\beta$-expressing cells were cultured in the presence or absence of 10 $\mu$M lactacystin for 3 h. Each stable cell line was solubilized in 1 mL of lysis buffer at 4 °C for 30 min. The cell lysate (1 mg) was incubated with anti-α-subunit antibody HK9, and Protein A-coated beads. The precipitated preparations were separated on an SDS−polyacrylamide gel and subjected to the Western blotting with an anti-polyubiquitin antibody. Ub-α shown on the right side represents the ubiquitinated α-subunit. (B) Western blots with an anti-α-subunit antibody of the anti-polyubiquitin immunoprecipitates from cell lysate samples. (C) Western blot with an anti-α-subunit antibody of the anti-α-subunit immunoprecipitates from cell lysate samples. The α-expressing cell preparations precipitated with an anti-α-subunit antibody HK9 were subjected to the Western blotting with an anti-α-subunit antibody 1H9. α and Ub-α represent the unubiquitinated and ubiquitinated α-subunits, respectively.

and indicate that the α-subunit in the α+L-1,2,3-expressing cells was modified with polyubiquitin.

*Polyubiquitination of the* α*-Subunit in the Stable Cell Lines Coexpressing the Wild-Type* α*- and Mutant* $\beta$*-Subunits.* Figure 9 shows the Western blots with an anti-polyubiquitin antibody of anti-α-subunit immunoprecipitates from lysates of the α+L-1,2,3-expressing cells and the $\alpha+\beta$-expressing cells. In the absence of lactacystin, polyubiquitination of the α-subunit was observed as a faint smeary band with a molecular mass of 100−300 kDa in the cell lysate prepared from the α+L-1,2,3-expressing cells. Detection of poly-ubiquitinated α-subunit was significantly enhanced when this cell line was cultured in the presence of lactacystin. The

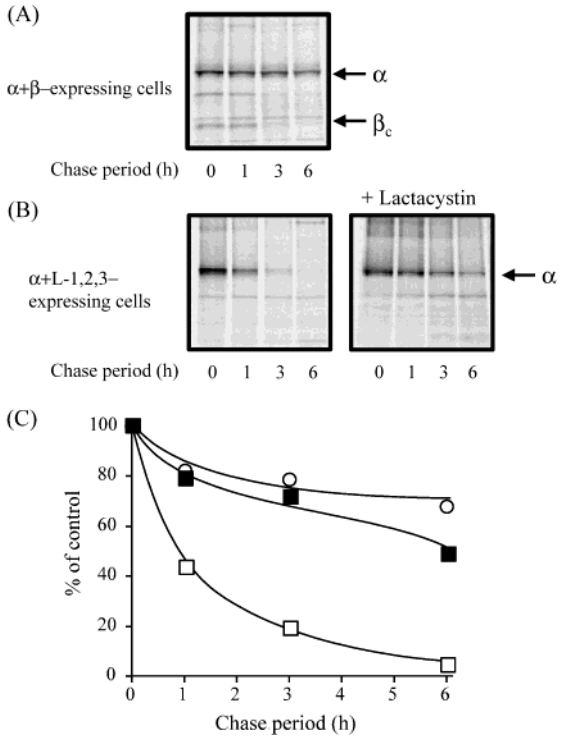


FIGURE 6: Pulse chase labeling of the α+*β*-expressing cells (A) and the α+L-1,2,3-expressing cells (B), followed by the immunoprecipitation with an anti-α-subunit antibody. Stable cell lines cultured on collagen-coated six-well plates were labeled for 60 min with [$^{35}$S]-Met, Cys Labeling mixture (EXPRESS), followed by a chase in the complete medium for indicated periods in the presence or absence of 10 *μ*M lactacystin. Cells were solubilized in 500 *μ*L of lysis buffer at 4 °C for 30 min. After centrifugation of the sample, the supernatant was incubated with anti-α-subunit antibody 1H9, and ImmunoPure Immobilized Protein A at 4 °C for 12 h. After centrifugation of the sample, the pellet was incubated in the sample buffer for SDS−polyacrylamide gel electrophoresis, separated on the gel, and visualized by digital autoradiography. α and $\beta_c$ represent the α-subunit and the *β*-subunit modified with high mannose-type carbohydrate chains, respectively. (C) Quantification of the intensity of the bands representing the α-subunit shown in panels A and B. The quantity of the α-subunit after each chase period was counted from the density of the digital autoradiography, and expressed as the percentage of that at 0 time in each condition. ○ represents the quantity of the α-subunit in the α+*β*-expressing cells in the absence of lactacystin. □ and ■ represent the quantity of the α-subunit in the α+ L-1,2,3-expressing cells in the absence and presence of lactacystin, respectively.

extent of polyubiquitination was much higher in the α+L-1,2,3-expressing cells than that in the α+*β*-expressing cells.

## DISCUSSION

The gastric $H^+$,$K^+$-ATPase α-subunit leaves the ER and travels to the cell surface only in the presence of the *β*-subunit, whereas the *β*-subunit can travel to the cell surface even in the absence of α-subunit. In the present study, we found that the ubiquitin/proteasome system is very important in degradation of unassembled α-subunits, but not involved in degradation of unassembled *β*-subunits. Therefore, this system seems to be important in the regulation of the number of the α- and *β*-subunits in the ER to control the cell surface expression of functional α/*β* holoenzymes. Similar phenomena were reported for quality control of oligomeric membrane proteins such as $Na^+$,$K^+$-ATPase, ENaC, and T-cell receptor (*30*, *31*, *42*).

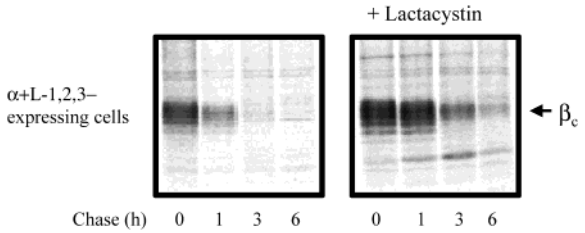


FIGURE 7: Pulse chase labeling of the α+L-1,2,3-expressing cells, followed by the immunoprecipitation with an anti-*β*-subunit antibody. Stable cell lines cultured on collagen-coated six-well plates were labeled for 60 min with [$^{35}$S]-Met, Cys Labeling mixture (EXPRESS), followed by a chase in the complete medium for indicated periods in the absence and presence of 10 *μ*M lactacystin. Cells were solubilized in 500 *μ*L of lysis buffer at 4 °C for 30 min. After centrifugation of the sample, the supernatant was incubated with anti *β*-subunit antibody 2B6, and ImmunoPure Immobilized Protein A at 4 °C for 12 h. After centrifugation of the sample, the pellet was incubated in the sample buffer for SDS−polyacrylamide gel electrophoresis, separated on a gel, and visualized by digital autoradiography.

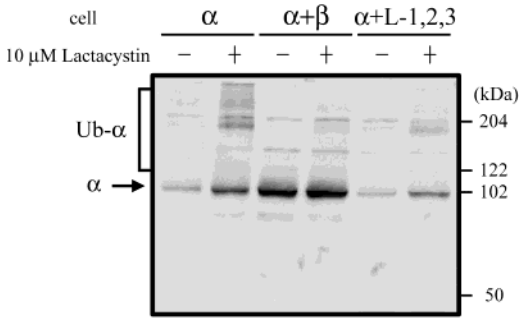


FIGURE 8: Western blots with an anti-α-subunit antibody of the cell lysate preparations prepared from the α-expressing, α+*β*-expressing, and α+L-1,2,3-expressing cells cultured in the presence or absence of 10 *μ*M lactacystin for 3 h. Each stable cell line was incubated in 1 mL of lysis buffer at 4 °C for 30 min. The cell lysate (30 *μ*g) was separated on an SDS−polyacrylamide gel and subjected to Western blotting with anti-α-subunit antibody Ab1024. α and Ub-α shown on the left side represent the unubiquitinated and ubiquitinated α-subunits, respectively.

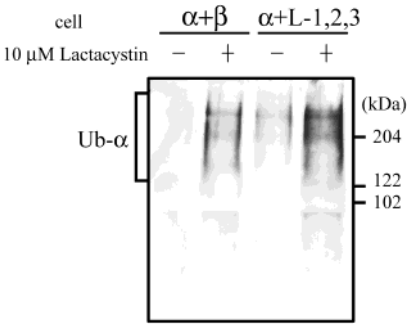


FIGURE 9: Western blots with an anti-polyubiquitin antibody of the anti-α-subunit immunoprecipitates from lysate of stable cell lines. The α+*β*-expressing and α+L-1,2,3-expressing cells were cultured in the presence or absence of 10 *μ*M lactacystin for 3 h. Each stable cell line was solubilized in 1 mL of lysis buffer at 4 °C for 30 min. The cell lysate (1 mg) was incubated with anti-α-subunit antibody HK9, and Protein A-coated beads. The precipitated preparations were separated on the SDS−polyacrylamide gel and subjected to the Western blotting with an anti-polyubiquitin antibody.

$Na^+$,$K^+$-ATPase belongs to the same P-type ATPase family as the gastric $H^+$,$K^+$-ATPase. This pump also consists of the catalytic α- and noncatalytic *β*-subunits. Association of these two subunits and proper folding in the ER are

necessary for the cell surface delivery, whereas unassembled α-subunits are retained in the ER and degraded (*43*−*45*). When either the α1 or α2 subunit of $Na^+,K^+$-ATPase was overexpressed in COS-7 cells, it was modified with chains of ubiquitin polymer. However, the *β*-subunit was not polyubiquitinated (*31*). These results are in good agreement with our present finding with the gastric $H^+,K^+$-ATPase. Degradation of the unassembled $Na^+,K^+$-ATPase α-subunit and its protection by the *β*-subunit were also studied in *Xenopus* oocyte expression system (*46*). The authors reported that in the unassembled α-subunit poor membrane insertion efficiency of the C-terminal transmembrane segments (M5, M7, and M9) resulted in transient exposure of degradation signals to the cytoplasmic side. No degradation signal was found in the four N-terminal transmembrane segments (M1 to M4) and the large cytoplasmic loop in the unassembled α-subunit. It is noteworthy that one of the putative degradation signal, Pro−Leu−Pro, located in the extracellular loop between the M5 and M6 segments is highly conserved in the $Na^+,K^+$-ATPase and the gastric $H^+,K^+$-ATPase. This Pro−Leu−Pro sequence may be active as the degradation signal in the unassembled $H^+,K^+$-ATPase α-subunit. They also found that degradation of the unassembled $Na^+,K^+$-ATPase α-subunit was partially inhibited by lactacystin (*46*).

In High Five insect cells, the $Na^+,K^+$-ATPase α-subunit, expressed alone, is degraded in the ER, whereas the *β*-subunit is processed normally and delivered to the cell surface (*45*).

ENaC is a heteromultimeric protein consisting of three kinds of subunits, α, *β*, and *γ*. Cell surface expression of this channel is highly regulated. In the Madin-Darby canine kidney (MDCK) expression system and in HEK-293 cells, the α- and *γ*-subunits were ubiquitinated, whereas the *β*-subunit was not. The expression level of these three subunits increased in the presence of lactacystin. These results suggest that the unassembled α- and *γ*-subunits are ubiquitinated and degraded by the proteasome (*30*). Biosynthesis and processing of ENaC was also studied in *Xenopus* oocyte expression system (*47*). Each subunit of the ENaC expressed individually was rapidly degraded. This degradation was blocked in the presence of lactacystin. However, coexpression of the α-, *β*-, and *γ*-subunits in a single cell stabilized each subunit in the ER. Only a small fraction of the total channel population was expressed at the cell surface. These results support the notion that multimeric proteins are stabilized by association with the correct partners.

It should be noted that ubiquitination of mammalian and yeast plasma membrane proteins (membrane receptors, channels, and transporters) leads to their internalization from the cell surface, followed by degradation in the lysosome (*48*). An assembled ENaC (α,*β*,*γ*) complex is also ubiquitinated and targeted for lysosomal degradation (*30*). This process is important for the regulation of the ion channel activity of ENaC. In the case of Liddle's syndrome, mutant ENaCs have defects in their carboxy-terminal PY motif, which is important for the interaction with the WW motif of the Nedd4 protein involved in ubiquitination. Therefore, the mutant ENaCs are stabilized at the cell surface, resulting in abnormally increased channel activity (*49*, *50*).

The gastric proton pump is also internalized from the cell surface after cessation of acid secretion. It is reported that the *β*-subunit contains a Tyr-based sorting signal for internalization (*51*). At present, however, it is not clear whether ubiquitination of the gastric proton pump is also involved in internalization from the cell surface and degradation in the lysosome.

In conclusion, we found that an unassembled α-subunit of the gastric proton pump is retained in the ER and ubiquitinated. The ubiquitin/proteasome system is involved in degradation of unassembled α-subunits in the ER to control the cell surface expression of functional α/*β* holoenzymes.

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