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Roles of 17-AAG-induced molecular chaperones and Rma1 E3 ubiquitin ligase in folding and degradation of Pendrin

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ABSTRACT

Pendrin is a transmembrane chloride/anion exchanger highly expressed in thyroid, kidney, and inner ear. Endoplasmic reticulum (ER)-retention of improperly folded Pendrin mutants is considered as the major cause for Pendred syndrome. However, the folding and degradation mechanisms of Pendrin are poorly understood. Here, we report that treatment of 17-AAG, an Hsp90 inhibitor, facilitates the folding of Pendrin through heat shock transcription factor 1 (Hsf1)-dependent induction of molecular chaperones. Furthermore, we demonstrate that Rma1, an E3 ubiquitin ligase localized in the ER membrane, is involved in Pendrin degradation.

1. Introduction

Pendrin is a transmembrane protein exchanging extracellular Cl^- against intracellular monovalent anions such as I^-, HCO_3^-, OH^-, and formate in the apical region of thyroid folliculocytes, kidney cortex, and inner ear [1–3]. Mutations in *SLC26A4* gene encoding Pendrin cause Pendred syndrome characterized by recessive congenital hearing impairment, thyroid goiter, and partial defects in iodide organification [4,5]. Overall Pendred syndrome comprises up to 10% of all congenital hearing impairments [6–8].

Retention of improperly folded Pendrin mutants in the endoplasmic reticulum (ER) has been suggested as the major pathological mechanism for Pendred syndrome [9,10]. Each Pendrin mutant shows a different level of folding defect, which can be inferred by its cellular localization, N-glycosylation level, and the degree of folding-rescue by low temperature incubation [11]. Like other transmembrane proteins, ER-retained misfolded Pendrin is believed to be degraded by ER-associated degradation (ERAD), which involves ubiquitylation, retro-translocation to the cytoplasm, and eventual degradation by the proteasome [12,13]. However, the molecular details of folding and degradation of Pendrin have not yet been investigated.

Here, we report that inhibition of Hsp90 activity by 17-AAG (17-allylamino-17-demethoxygeldanamycin) treatment increases the stability of Pendrin through Hsf1-dependent induction of various molecular chaperones. In addition, ER-resident E3 ubiquitin ligase Rma1 is shown to play an important role in Pendrin degradation. However, cytosolic ubiquitin ligase CHIP seems to exert little effect on Pendrin degradation. This is a first study to understand the roles of molecular chaperones and ubiquitin ligases in folding and degradation of Pendrin, which might contribute to developing strategies for curing Pendred syndrome.

2. Materials and methods

2.1. Plasmids

pCMV-Myc vectors containing cDNAs of Pendrin (*SLC26A4*) wild type and mutants (M147V, L236P, and H723R) were kindly provided by Dr. M.G. Lee (Yonsei University, College of Medicine, Seoul, Korea) [11]. To generate plasmids containing Pendrin truncation mutants, PCR-amplified Pendrin cDNA fragments (Pendrin$_{1-166}$, Pendrin$_{1-334}$, and Pendirn$_{1-522}$), were cloned into the XhoI and NotI sites of pCMV-Myc. Human Rma1 cDNA clones were generated by RT-PCR using mRNA isolated from HEK293 cells. The PCR products were cloned into the KpnI and NotI sites of

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; ERAD, ER-associated degradation; Hsf1, heat shock transcription factor 1; TPR, tetratricopeptide repeat; 17-AAG, 17-allylamino-17-demethoxygeldanamycin

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pcDNA3.1-NFlag, which was generated by inserting a flag-tag sequence at the NheI site of pcDNA3.1 (Invitrogen).

2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) and HeLa cells were maintained in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Plasmids or siRNA duplexes were transfected into cells by using Lipofectamine2000 (Invitrogen). For siRNA, cells were transfected with 100 pmol of siRNA duplex targeted to Hsf1 (5′-CAGGUUGUUCAUAGUCAGAAU-3′), Rma1 (5′-GCGACCUUCGAAUGUAAUA-3′) and CHIP (5′-UGCCGCCACUAUCUGUGUAAU-3′). Scrambled siRNA duplex (Bioneer) was used as a control.

2.3. Immunoblotting

Harvested HEK293 and HeLa cells were lysed with a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 2 mM EDTA, and 1% NP-40 supplemented with 1 mM PMSF, and 1/1000 protease inhibitor cocktail (Calbiochem) for 30 min at 4 °C. Following SDS–PAGE, immunoblotting was performed by using antibodies against Myc (Santa Cruz Biotechnology), Flag (Cell Signalling), β-tubulin (Santa Cruz Biotechnology), Hsp70 (Stressgen), Hsf1 (Cell Signalling), CHIP (Cell Signalling), Rma1 (Santa Cruz Biotechnology), and the chemiluminescence signals were detected and quantified by chemiluminescence image equipment (Syngene).

2.4. Immunofluorescence microscopy

HeLa cells expressing Myc-Pendrin wild type and H723R were seeded into incubation chamber pretreated with poly D-lysine for 4 h. 24 h after transfection of the expression vectors, 50 nM of 17-AAG was treated using 0.25% DMSO as a control. After 24 h, cells were fixed by 4% paraformaldehyde for 20 min at 4 °C, and then 0.25% Triton X-100 was added into the fixed cells for antibody permeability. Primary antibody against Myc was incubated for 2 h at 4 °C, and then FITC-labeled secondary antibody was added. After staining nucleus with DAPI for 1 min, cellular localizations of FITC-labeled Myc-Pendrin were analyzed by confocal microscope (Nikon, Eclipse TE 300).

3. Results

3.1. Treatment of 17-AAG, an Hsp90 inhibitor, increases the stability of Pendrin

One of the predicted topologies of Pendrin suggests 12 transmembrane domains with both N-terminal and C-terminal ends facing cytosol, although it needs further experimental verification [8,14,15], and Pendrin has two N-glycosylation sites at Asn167 and Asn172 residues [16] (Fig. 1). As previously reported [11], Myc-tagged Pendrin transiently expressed in HEK293 cells showed at least three bands on SDS–PAGE, which are designated as A, B, and C forms (Fig. 2A). The A form is predicted to be an unglycosylated form of Pendrin. The B form is immaturely glycosylated Pendrin in the ER and the C form represents maturely glycosylated Pendrin on the plasma membrane.

Hsp90 has been shown to play an important role in the folding of cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel whose folding defects are implicated in cystic fibrosis [17]. Therefore, we asked whether Hsp90 is also involved in the folding of Pendrin. To address this question, we investigated the effect of Hsp90 inhibitor, 17-AAG, on the steady state level of Pendrin protein. Pendrin was expected to be degraded by 17-AAG treatment if Pendrin is a client protein of Hsp90 like CFTR. However, in contrast to our expectation, when HEK293 cells expressing Myc-Pendrin were treated with 17-AAG for 24 h, the A and B forms, but not C form, were stabilized in a dose dependent manner (Fig. 2A and E). Induction of Hsp70 by 17-AAG was monitored as an indicator of Hsf1 activation. Since Hsp90 inhibits Hsf1 transcription factor, inhibition of Hsp90 activates Hsf1, which in turn leads to the induction of Hsf1 target genes most of which encode molecular chaperones. 17-AAG treatment did not affect the transcript level of Pendrin, which was detected by qRT-PCR (Supplementary Fig. 1). Moreover, Myc-PP5 expressed from the same CMV promoter-controlled expression vector did not show any change in PP5 protein levels upon 17-AAG treatment (Supplementary Fig. 2). Therefore, 17-AAG-dependent increase in the Pendrin protein levels seems not to be caused by the effect of 17-AAG on transcription or translation of the Pendrin gene.

We also examined the effect of 17-AAG on three Pendrin mutants (M147V, L236P, and H723R) which were shown to have different levels of folding defects [11]. Pendrin M147V and H723R showed reduced levels of C forms compared with wild type

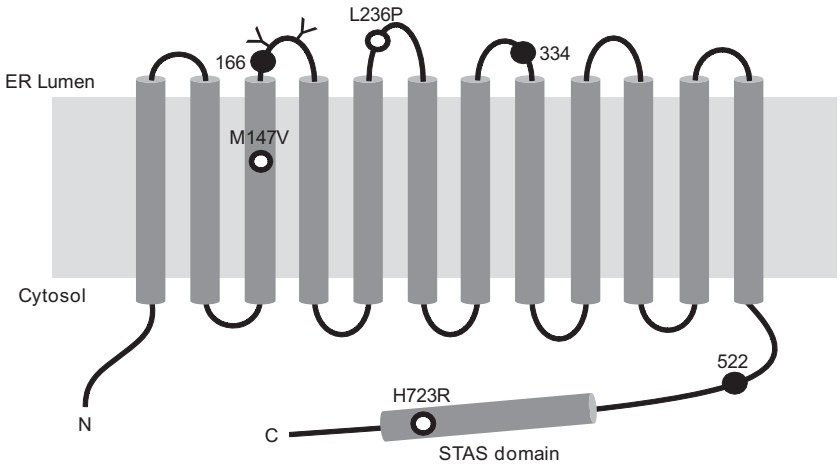

Fig. 1. Schematic diagram of the putative topology of Pendrin. The putative topology of Pendrin is shown as previously reported [16]. N-glycosylation sites are denoted as 'Y'. The mutation sites of Pendrin M147V, L236P, and H723R are indicated as open circles, and closed circles designate the locations where stop codons are introduced to generate truncation mutants. STAS is a sulfate transporter and antisigma factor antagonist domain.

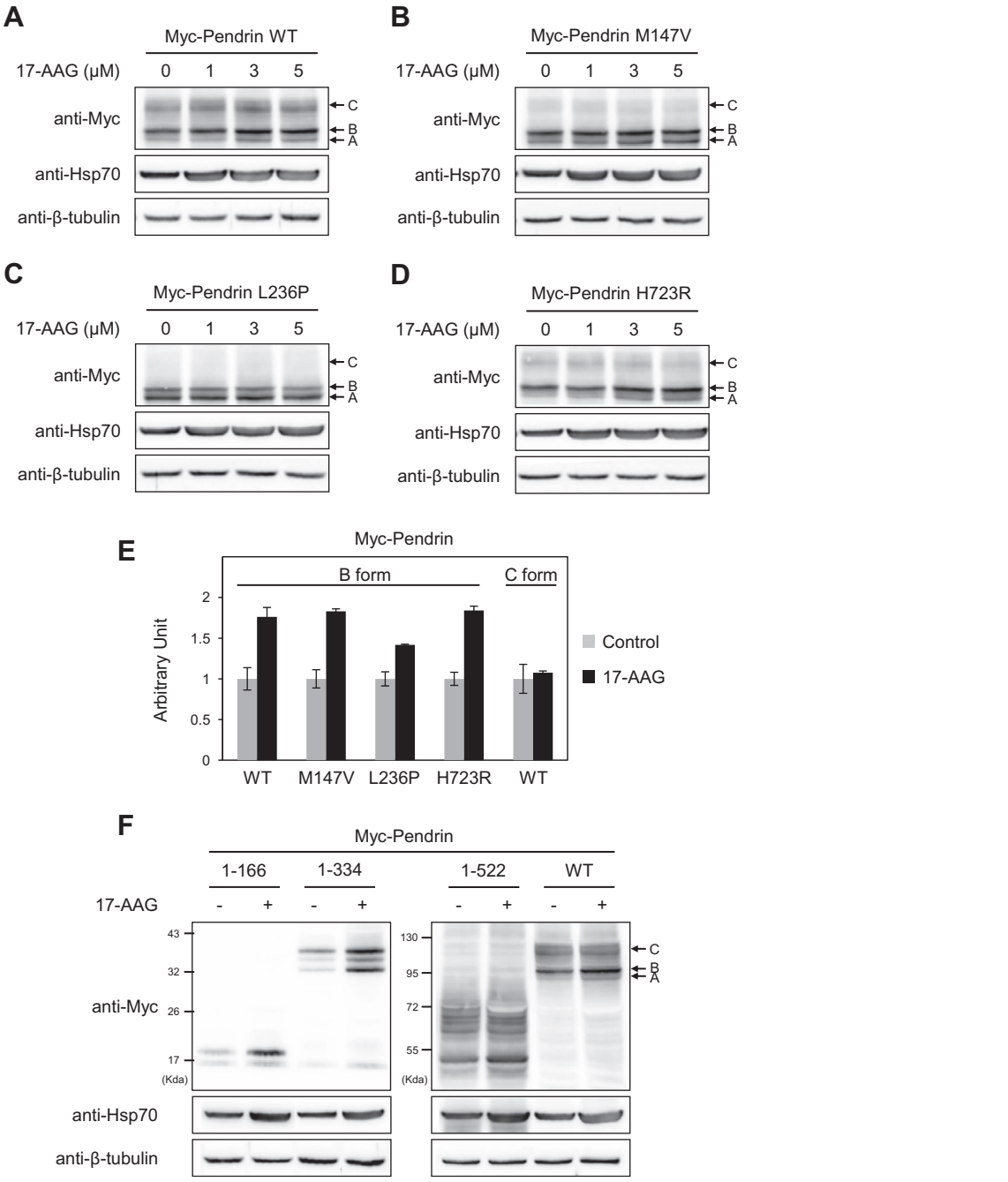

Fig. 2. Stabilization of Pendrin by 17-AAG in HEK293 cells. Myc-tagged Pendrin wild type (A) and mutants, M147V (B), L236P (C), and H723R (D) were transiently expressed in HEK293 cells, and the indicated amount of 17-AAG was treated for 24 h after transfection. 0.25% DMSO was treated as a control. Expression levels of Myc-Pendrin, Hsp70, and β-tubulin were detected by immunoblotting. A, B, and C indicate unglycosylated, immaturely glycosylated, and maturely glycosylated forms of Pendrin, respectively. (E) The relative amounts of wild type and mutant Pendrin B forms, and wild type C form after treatment with 0.25% DMSO (gray bar) or 5 μM 17-AAG (black bar) were quantified from three independent experiments. The quantification values of Pendrin were normalized to β-tubulin. (F) Stabilization of Pendrin truncation mutants by 17-AAG. Transfected cells were treated for 24 h with 5 μM 17-AAG or 0.25% DMSO as a control.

(Fig. 2B and D), and L236P, having the most severe folding defects, did not generate any mature C form and showed higher level of A form compared with wild type or other mutants (Fig. 2C). 17-AAG treatment stabilized the A and B forms of Pendrin M147V and H723R, but not L236P (Fig. 2B–E). Neither wild type nor the mutants showed noticeable changes in the stability of C forms upon 17-AAG treatment. Therefore, Hsp90 inhibition might induce partial folding-rescue of Pendrin proteins, and its effect seems to be different depending on the folding status of Pendrin.

To map the specific regions of Pendrin where the stability is affected by 17-AAG, Pendrin truncation mutants were generated and expressed in HEK293 cells (Fig. 1). 17-AAG treatment stabilized all these truncation mutants, indicating that 17-AAG affects Pendrin stability from the early stage of Pendrin synthesis (Fig. 2F).

Since different cell types have different cellular environments including the chaperone system [18], the effect of 17-AAG treatment on Pendrin stability was also examined in human cervical cancer cell line HeLa cells. Myc-tagged Pendrin, transiently expressed in HeLa cells, exhibited higher levels of C form than either the A or B form, and 17-AAG treatment led to a prominent increase in the C form (Fig. 3A). These results might be caused by higher folding capacity of HeLa cells for Pendrin compared with HEK293

cells. On the other hand, lower Pendrin expression levels derived from lower transfection efficiency of HeLa than HEK293 cells could also possibly contribute to the efficient folding of Pendrin in HeLa cells.

We also examined the effects of 17-AAG on the folding of Pendrin mutants, M147V and H723R, in HeLa cells. 17-AAG treatment not only stabilized the A and B forms of Pendrin H723R, but also partly rescued this mutant to the mature C form (Fig. 3B). Whereas, Pendrin M147V, having stronger folding defects than H723R, failed to generate the C form from the increased pool of A and B forms (Fig. 3B). Next, we tested whether the C form of Pendrin H723R increased by 17-AAG treatment actually reflected the proteins reached to the plasma membrane. Indirect immunofluorescence microscopy revealed that Pendrin wild type was localized in the plasma membrane as well as distributed throughout the cytosol as small aggregates (Fig. 3C). In contrast, Pendrin H723R was retained in the perinuclear region in the form of aggregates, reflecting the ER-retention of the misfolded mutant proteins (Fig. 3C). 17-AAG treatment noticeably induced plasma membrane localization of Pendrin H723R, whereas the membrane localization of wild type Pendrin was not significantly changed by 17-AAG (Fig. 3C). Therefore, depending on cellular environment, 17-AAG treatment can facilitate the folding and exit of Pendrin from the ER to generate a maturely glycosylated form. These results suggest that the 17-AAG-dependent increase in Pendrin stability might involve the improvement of protein folding rather than the simple accumulation of misfolded proteins by inhibiting their degradation.

3.2. Hsf1 mediates the 17-AAG-dependent stabilization of Pendrin

Since 17-AAG treatment seems to assist Pendrin folding, we hypothesized that the 17-AAG effect might be mediated by the induction of molecular chaperones through the activation of Hsf1 rather than by a direct effect of the reduced Hsp90 chaperone activity. To test this possibility, we investigated the effect of Hsf1 depletion on the 17-AAG-dependent stabilization of Pendrin. When HEK293 cells were co-transfected with Hsf1 siRNA and Pendrin expression vector, 17-AAG-dependent Pendrin stabilization was diminished, concomitant with the reduction of Hsp70 induction (Fig. 4). Upon 17-AAG treatment, the Pendrin B form was stabilized by only 1.5-fold in Hsf1 knockdown cells compared with a stabilization by 2.5-fold in cells transfected with scrambled siRNA. Therefore, the Hsf1-dependent induction of various molecular chaperones might be mainly responsible for the Pendrin stabiliza-

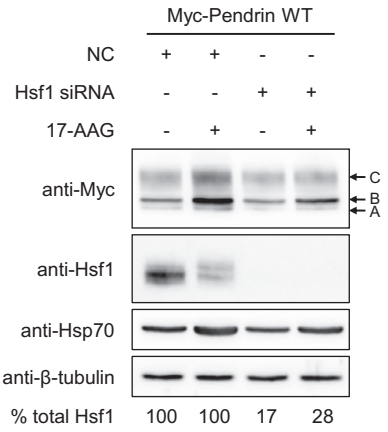

Fig. 4. Hsf1-dependent induction of molecular chaperones is responsible for Pendrin stabilization upon 17-AAG treatment. HEK293 cells were co-transfected with Myc-Pendrin expression vector and Hsf1 siRNA duplex. Scrambled siRNA was used as a negative control (NC). After 24 h of transfection, cells were treated with 5 μM 17-AAG for 24 h and protein expression levels were analyzed by immunoblotting. The relative amounts of Hsf1 normalized to β-tubulin are indicated.

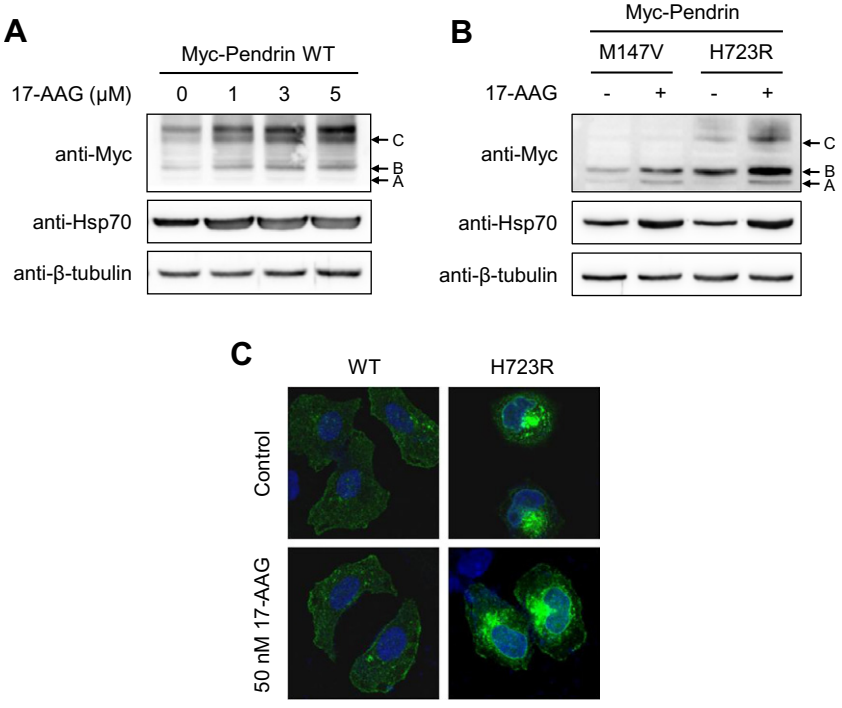

Fig. 3. Effect of 17-AAG on Pendrin stability in HeLa cells. (A) Myc-Pendrin was transiently expressed in HeLa cells and the expression levels of Pendrin were detected after treatment of the indicated amount of 17-AAG for 24 h. (B) 5 μM of 17-AAG was treated for 24 h to HeLa cells transfected with expression vectors for Myc-Pendrin M147V or H723R. (C) Localizations of Myc-Pendrin wild type and H723R were detected by immunofluorescence microscopy with or without the treatment of 50 nM 17-AAG for 24 h (green). Nucleus was stained with DAPI (blue).

tion by 17-AAG. To identify specific molecular chaperones responsible for Pendrin stabilization, we selected the major molecular chaperone Hsp70 and its Hsp40 cochaperones, Hdj1 and Hdj2 as candidates among the known Hsf1 targets. However, overexpression of Hsp70, Hdj1, and Hdj2 alone or in combination did not induce significant changes in Pendrin stability (data not shown). Therefore, the 17-AAG effect might reflect coordinated actions of multiple chaperones yet to be identified.

3.3. E3 ubiquitin ligase Rma1 is involved in Pendrin degradation

Misfolded Pendrin is believed to be degraded by ERAD, but E3 ubiquitin ligases mediating the process have not yet been identified. Among the known E3 ubiquitin ligases involved in ERAD, we first examined whether Rma1 functions in Pendrin degradation. Rma1, which forms ER membrane-associated ubiquitin ligase complex with Ubc6e and Derlin-1, has been shown to promote proteasomal degradation of CFTR [19]. Overexpression of Rma1 reduced the steady state levels of Pendrin WT and H723R, but not M147V and L236P (Fig. 5A and C). In addition, siRNA reduction of Rma1 by around 49% slightly increased the amount of Pendrin wild type B form, suggesting that Rma1 is involved in Pendrin degradation (Fig. 5B and C). Pendrin mutants having higher folding defects seem not to be susceptible to the changes in Rma1 levels under our experimental conditions. To find out specific regions of Pendrin where Rma1 recognizes for degradation, we also investigated the effect of Rma1 on the stability of Pendrin truncation mutants. Overexpression of Rma1 decreased the protein levels of $Pendrin_{1-334}$ and $Pendrin_{1-552}$ as well as wild type, but not $Pendrin_{1-166}$ (Fig. 5D). Therefore, Rma1 might recognize folding defects after the synthesis of the third transmembrane domain (Fig. 1).

Next, we investigated the role for CHIP in Pendrin degradation. CHIP is a cytosolic E3 ubiquitin ligase which interacts with Hsp70/Hsc70 and Hsp90 through its tetratricopeptide repeat (TPR) motifs, and is also known to regulate CFTR degradation [19,20]. However, neither overexpression nor depletion of CHIP significantly changed the steady state levels of Pendrin (Fig. 6A–C). Therefore, unlike Rma1, CHIP might play little role in Pendrin degradation.

4. Discussion

It has been suggested that ER-retention of improperly folded Pendrin is the major cause of Pendred syndrome. Although Pendred disease comprises up to 10% of all congenital hearing deficiencies, the folding and degradation mechanisms of Pendrin are largely unknown. Our study is a first attempt to elucidate the roles of molecular chaperones and E3 ubiquitin ligases involved in Pendrin folding and degradation.

Initially, we used 17-AAG, an Hsp90 inhibitor, to test whether Pendrin is a client protein of Hsp90 similar to CFTR. However, Pendrin was stabilized by 17-AAG treatment unlike typical Hsp90 client proteins which are degraded upon Hsp90 inhibition. The Pendrin-stabilization effect of 17-AAG was abolished by siRNA reduction of Hsf1, indicating that induction of various molecular chaperones is mainly responsible for the 17-AAG-dependent Pendrin stabilization. Since overexpression of Hsp70 and its Hsp40 cochaperones, Hdj1 and Hdj2, failed to deliver the stabilization ef-

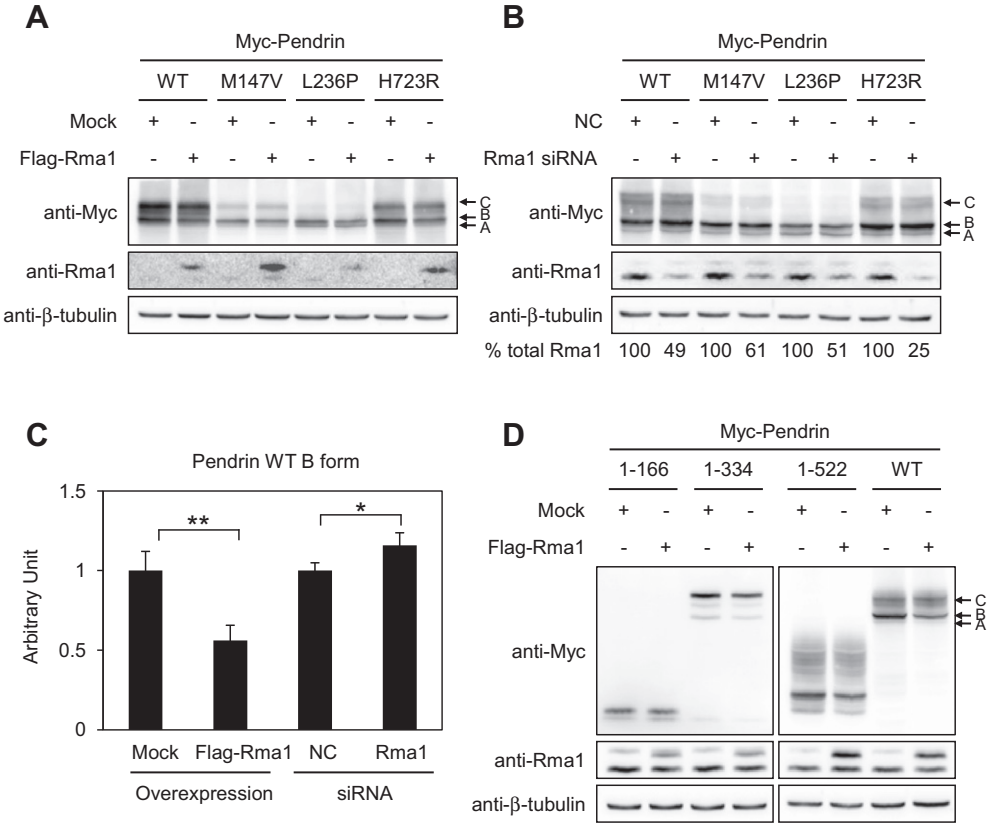

Fig. 5. Effect of Rma1 on Pendrin stability. (A and D) Flag-Rma1 was overexpressed with Myc-Pendrin or its mutants in HEK293 cells. After 24 h of transfection, protein levels were analyzed by immunoblotting. (B) Cellular Rma1 was depleted by Rma1 siRNA duplex 24 h before the transfection of expression vector for Myc-Pendrin wild type or its mutants. After 24 h of further incubation, protein expression levels were detected by immunoblotting. The relative amounts of Rma1 normalized to β-tubulin are indicated. (C) The relative amounts of Pendrin WT B forms upon overexpression or depletion of Rma1 were quantified from three independent experiments. $^{**}P < 0.01$; $^{*}P < 0.05$ (unpaired Student's *t*-test).

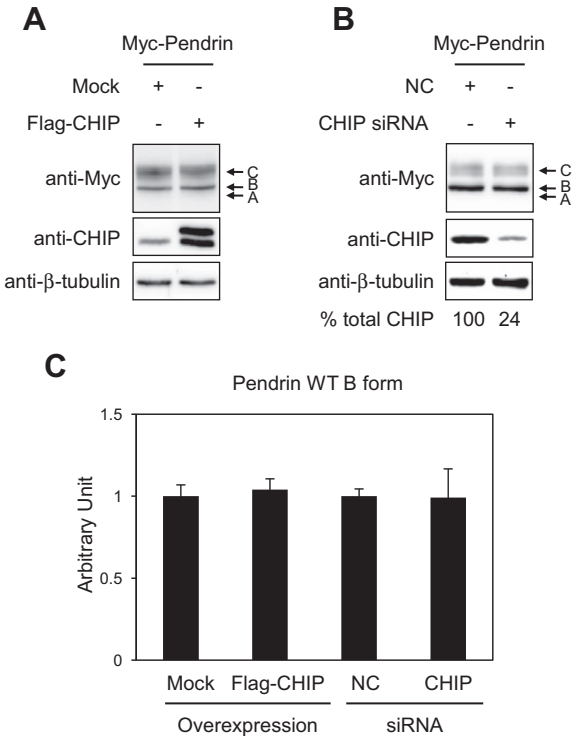

Fig. 6. Effect of CHIP on Pendrin stability. (A) HEK293 cells were co-transfected with expression vectors for Flag-CHIP and Myc-Pendrin for 24 h, and protein expression levels were detected by immunoblotting. (B) Cellular CHIP was depleted by siRNA for 24 h before the transfection of expression vector for Myc-Pendrin, and then further incubated for 24 h to detect protein expression levels. The relative amounts of CHIP normalized to β-tubulin are indicated. (C) The relative amounts of Pendrin WT B forms upon overexpression or depletion of CHIP were quantified from three independent experiments.

fect of Pendrin unlike the previous report demonstrating their roles in CFTR stabilization [21], balanced actions of other chaperones might be responsible for proper Pendrin folding.

Pendrin expressed in HeLa cells displayed higher levels of Pendrin folding and processing than that expressed in HEK293 cells, possibly reflecting the differences in folding or cellular trafficking capacity of each cell type. Although 17-AAG could hardly rescue the B form of wild type or mutant Pendrin to the C form in HEK293 cells, 17-AAG could partially rescue the folding defects of H723R mutant to generate the membrane-localized C form in HeLa cells. These results demonstrate that Pendrin folding and trafficking to the plasma membrane can be altered by changes in the cellular environment, and Hsp90 inhibitors can facilitate Pendrin folding by optimizing the cellular folding capacity. Previously, low temperature incubation, protein synthesis inhibitors, and chemical chaperones were also shown to assist Pendrin folding [11,13]. Although Hsp90 inhibitors are mainly considered as promising anti-cancer drugs targeting many oncogenic Hsp90 client proteins, they also have a pharmacological potential to treat neurodegenerative diseases by acting as inducers of molecular chaperones [22,23]. It has been shown that Hsp90 inhibitors can alleviate neurodegenerative diseases such as Huntingtun's disease, tauopathy, and Parkinson's disease by inhibiting protein aggregation [24–26]. Our results suggest that Hsp90 inhibitors might also have a potential to be employed to cure Pendred syndrome.

We examined the roles for Rma1 and CHIP E3 ubiquitin ligases, which have been known to degrade improperly folded CFTR, in degradation of Pendrin [19,20]. Our Rma1 overexpression and siRNA experiments suggest that the ER-resident Rma1 may be involved in Pendrin degradation by recognizing its folding defects after the synthesis of the third transmembrane domain. On the other hand, it was difficult to detect clear effect of CHIP on Pendrin degradation. Compared with CFTR, Pendrin is predicted to have smaller domains exposed to cytosol, which might be partly related to the minor effects of the cytosolic ubiquitin ligase CHIP and cytosolic molecular chaperone Hsp90 on Pendrin stability.

In summary, we found that 17-AAG, an Hsp90 inhibitor, has a therapeutic potential to rescue Pendrin folding defects through the activation of Hsf1. The folding of Pendrin might be regulated by the balanced actions of multiple components of chaperone system and ERAD machinery, which are waiting for further investigation. Our study to understand the molecular mechanisms of Pendrin folding and degradation would serve as a first step to develop curing strategies for Pendred syndrome.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.06.023.

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