Conserved Amino Acid Residues in the COOH-terminal Tail Are Indispensable for the Correct Folding and Localization and Enzyme Activity of Neutral Ceramidase*

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Several lines of evidence suggest that neutral ceramidase is involved in the regulation of ceramide-mediated signaling. Recently, the enzymes from mouse and rat were found to be localized at plasma membranes as a type II integral membrane protein, occasionally being detached from the cells after proteolytic processing of the NH2-terminal anchoring region (Tani, M., Iida, H., and Ito, M. (2003) J. Biol. Chem. 278, 10523–10530). We report here that conserved hydrophobic amino acid residues in the COOH-terminal tail are indispensable for the correct folding and localization, and enzyme activity of neutral ceramidase. Truncation of four, but not three, amino acid residues from the COOH terminus of rat neutral ceramidase resulted in a complete loss of enzyme activity as well as cell surface expression in HEK293 cells. Point mutation analysis revealed that Ile786, the 4th amino acid residue from the COOH terminus, and Phe786 are essential for the enzyme to function. The truncated and mutated enzymes were found to be retained in the endoplasmic reticulum (ER) and rapidly degraded without transportation to the Golgi apparatus. Treatment of the cells expressing the aberrant COOH-terminal enzyme with MG-132, a specific inhibitor for the proteasome, increased the accumulation of the enzyme in the ER, indicating that the misfolded enzyme was degraded by the proteasome. It was also found that the COOH-terminal tail was indispensable for the enzyme activity and correct folding and the NH2-terminal anchoring region contains a conserved motif in vertebrate enzymes, whereas the bacterial and invertebrate enzymes do not, and a mutant mammalian CDase lacking the mucin box was secreted into the medium instead of localizing at the cell surface.

Ceramide (N-acylsphingosine, Cer), sphingosine (Sph), and sphingosine 1-phosphate (S1P) have emerged as a new class of lipid biomodulators for various cell functions (1–3). Both Cer and Sph have been shown to induce growth arrest and apoptosis (1, 2), whereas S1P appears to promote cell growth and proliferation and suppress the apoptosis induced by Cer (3). Consequently, the balance of the cellular contents of Cer/Sph/S1P is thought to regulate the diversity of cellular responses.

Ceramidase (CDase, EC 3.5.1.23) is an enzyme that cleaves the N-acyl linkage of Cer to produce Sph and free fatty acid (4). Three categories of CDases: acid, neutral, and alkaline, are clearly distinguished not only by their catalytic pH optima, but also their primary structures (5). Neutral CDase, which shows an optimum pH of 6.5–8.5, has been cloned from bacteria (6), Drosophila (7), mouse (8), rat (9), and human (10). Accumulating evidence suggests that neutral CDase regulates the intracellular content of Cer and thereby Cer-mediated signaling. For example, an increase of Cer caused by the IL-1β-stimulated hydrolysis of SM was gradually normalized by up-regulation of neutral CDase as detected in mRNA and de novo synthesis levels after long term treatment with IL-1β, showing the function of neutral CDase as a cytoprotective enzyme in mesangial cells exposed to inflammatory stress (11). In contrast, nitric oxide (NO) down-regulates the protein expression of neutral CDase via a protein kinase C-dependent mechanism in renal mesangial cells, resulting in an increase of Cer after treatment of the cells with NO (12). Very recently, the targeted expression of neutral CDase was found to reverse the retinal degeneration by normalizing the Cer level in Drosophila arrestin and phospholipase C mutants (13). These results suggest that neutral CDases may be suitable targets in the therapeutic management of cytokine-induced inflammation and retinal degeneration.

Although the primary structure of neutral CDase is highly conserved from bacteria to mammals (8), the subcellular localization of the enzyme is quite different depending on its origin. Mammalian neutral CDases are present at the plasma membrane as a type II integral membrane protein, occasionally being detached from cells after proteolytic processing of the NH2-terminal anchoring region (14), whereas the bacterial and invertebrate enzymes are solely secretory proteins (7, 15). The discrepancy may stem from whether a Ser/Thr-rich domain (mucin box) is present downstream of the NH2-terminal signal anchor sequence of the enzyme, because all mammalian CDases possess a mucin box, whereas the bacterial and invertebrate enzymes do not, and a mutant mammalian CDase lacking the mucin box or possible O-glycosylation sites in the mucin box was secreted into the medium instead of localizing at the cell surface (14).

Very recently, a change in the COOH-terminal region of acid CDase was identified to be a novel mutation in Farber disease in which Cer is accumulated in lysosomes due to the inactivation of acid CDase (16). In the present study, we found that two
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conserved amino acid residues, Phe and Ile/Val, in the COOH-terminal domain were completely conserved in neutral CDases from bacteria to humans. Thus, we examine the role of the conserved COOH-terminal tail of neutral CDases using mammalian and bacterial enzymes. In conclusion, we demonstrated that two conserved amino acids in the COOH-terminal tail are indispensable for the correct folding and localization, and enzyme activity of neutral CDase.

EXPERIMENTAL PROCEDURES

Materials—Anti-Rab6 antibody was kindly provided by Dr. S. Tanaka (Shizuoka University, Japan). HRP-labeled anti-mouse IgG antibody was purchased from Nacalai Tesque (Japan). Cy3-labeled anti-mouse IgG antibody, anti-FLAG M2 antibody, and benzyl-GalNAc were from Sigma. ECL plus, and HRP- and Cy3-labeled anti-rabbit IgG antibodies were obtained from Amersham Biosciences. MG-132 (benzylcarbonyl-Leu-Leu-Leu-aldehyde) and anti-Myc antibody were purchased from Calbiochem and Invitrogen, respectively. Anti-neutral CDase antibody was raised in a rabbit using the recombinant rat CDase as the antigen (9). C12-NBD-Cer was prepared as described in Ref. 17. HEK293 cells (JCRB9068, established by F. L. Graham) were obtained from the Human Science Research Resource Bank. All reagents were of the highest purity available.

CDase Assay—The hydrolysis and reverse hydrolysis activities of neutral CDase were measured using C12-NBD-Cer (for the hydrolysis reaction) and NBD-dodecyl acid and Spb (for the reverse hydrolysis reaction) as substrates (18).

Plasmid Construction—The vector pcDNA3.1/Myc-His(+) containing a full-length rat neutral CDase gene tagged with Myc at the COOH terminus (wild-type CDase) was prepared as described previously (9). Wild-type CDase tagged with FLAG at the NH2 terminus and Myc at the COOH terminus (FLAG-tagged CDase) was constructed as reported (14). The truncation mutants were constructed by PCR as described below. COOH-terminal fragments were amplified with a 5’ primer (5’-AAAGCCTGACGTTCCGCATTCACC-3’) and a 3’ primer with a XhoI restriction site (double underlined) and a XhoI restriction site (underlined): 5’-AGACTCGAGCTTCAATGTTGACAGCAG-3’ (for 5 XhoI CDase), 5’-AGACTCGAGCTTTCAATGTTGACAGCAG-3’ (for 3 XhoI CDase), 5’-AGACTCGAGCTTTCAATGTTGACAGCAG-3’ (for 2 XhoI CDase), and 5’-AGACTCGAGCTTTCAATGTTGACAGCAG-3’ (for 1 XhoI CDase). These fragments were digested with EcoRI and XhoI, and subcloned into the vector pcDNA3.1/Myc-His(+) containing FLAG-tagged CDase cDNA. Site-directed mutagenesis of wild-type CDase was carried out by the amplification of COOH-terminal fragments with a 5’ primer (5’-TTGATGAAGCAAAGCCTG-3’) and a 3’ primer with a XhoI restriction site (underlined): 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 11 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 10 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 9 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 8 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 7 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 6 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 5 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 4 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 3 CDase), 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 2 CDase), and 5’-CCGCTCGAGAGTAGTGACAAATTCAAAAGGGGAA-3’ (for 1 CDase). (double underline shows the location of mutations). These fragments were digested with EcoRI and XhoI, and subcloned into the vector pcDNA3.1/Myc-His(+) containing wild-type CDase cDNA. To obtain a plasmid vector containing Pseudomonas neutral CDase and the mutant DNA, DNA fragments were prepared by PCR using a 5’ primer containing a Nhel site (5’-AAAGCCTGACGTTCAATGTTGACAGCAGTACC-3’) and a 3’ primer with a XhoI restriction site: 5’-TTTCTCTCGAGGAGGAGGGAGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAGGGGAG
Fig. 2. Expression of wild-type and COOH-terminal-truncated rat neutral CDases in HEK293 cells. A, time course for hydrolysis (a) and reverse hydrolysis (b) activities. HEK293 cells were transfected with a plasmid vector containing wild-type or truncated enzyme cDNA. The hydrolysis and reverse hydrolysis activities of the cell lysates were determined using C12-NBD-Cer (for the hydrolysis reaction) and Sph and NBD-dodecanoic acid (for the reverse reaction) as substrates. B, enzyme activity and protein expression of wild-type and truncated enzymes. The neutral CDase activity of the cell lysate or medium was measured using C12-NBD-Cer as a substrate, and the protein expression was determined by Western blotting using anti-FLAG (for cell lysates) and anti-neutral CDase antibodies (for culture supernatants).

**RESULTS**

**Alignment and Truncation of COOH-terminal Region of Neutral CDases**—Although the primary structure of neutral CDase is highly conserved from bacteria to humans, the enzyme is classified into two prototypes; bacteria/invertebrate and mammalian types. The latter possesses a Ser/Thr-rich mucin-like domain (mucin box) downstream of the NH2-terminal signal/anchor sequence while the former does not (Fig. 1A). The mucin box was shown to retain the enzyme on the plasma membranes as a type II integral membrane protein. Thus mucin box-deleted mutant enzymes and bacteria/invertebrate enzymes were found to be secreted into the culture medium (7, 14, 15). In this study, we found that two amino acid residues, Phe and Ile/Val, in the COOH-terminal tail were completely conserved in both two types of the enzyme (Fig. 1A). To address the role of the conserved COOH-terminal tail, COOH-terminal-truncated mutant enzymes lacking 11, 6, 5, 4, and 3 amino acid residues from the COOH terminus, respectively, were constructed and expressed in HEK293 cells (Fig. 2B, ∆11, ∆6, ∆5, ∆4, ∆3). Wild-type and truncated enzymes were tagged with FLAG at the NH2 terminus. As shown in Fig. 2A, all truncated CDases except ∆3 lost the enzyme activity as measured in terms of the hydrolysis (a) and synthesis (b) of Cer. The enzyme activity of wild-type and ∆3 CDases was found in cell lysates as well as the medium (Fig. 2B). Western blotting analysis of the cell lysate using anti-FLAG antibody revealed that the wild-type and ∆3 CDases showed two protein bands having a molecular mass of 133 kDa and 113 kDa (Fig. 2B). The 133-kDa protein seems to be a mature form in the Golgi and the 113-kDa protein, developing form in the ER (9). A 130-kDa mature form was also detected in the culture medium of wild-type and ∆3 CDases when stained with anti-neutral CDase antibody (Fig. 2B). This secretory CDase is likely to be generated by processing of the NH2-terminal signal/anchor sequence from the mature form (14). In contrast, no mature forms were detected in the cell lysate or medium of ∆11, ∆6, ∆5, and ∆4 enzymes. These results suggest that the truncated CDases except ∆3 were not transported from the ER to the Golgi and lost enzyme activity.

Next, we analyzed the subcellular localization of COOH-
The truncated enzyme Δ3 retained the activity whereas Δ4 did not. Therefore, Ile758 of the rat enzyme, the 4th amino acid residue from the COOH terminus, is likely to be integral to the enzyme activity and correct subcellular localization (Fig. 1A). In addition to Ile758, Phe756 is also conserved in all enzymes (Fig. 1A). Thus, we examined the effects of point mutations of Ile758 and Phe756 on enzyme activity and subcellular localization. All mutant and wild-type enzymes were tagged with Myc at the COOH terminus and expressed in HEK293 cells. As shown in Fig. 4A, replacement of Ile758 with Asp or Arg almost completely abolished the enzyme activity. On the other hand, no decrease in enzyme activity was observed when Ile758 was replaced with Val, which is conserved in all neutral CDases except the rat enzyme. Replacement of Ile758 with Phe decreased the enzyme activity to 20% in comparison with wild-type CDase. Western blotting analysis using anti-Myc antibody detected the 113-kDa ER form, but not the 133-kDa mature form, in the cell lysates when I758D and I758R mutants were overexpressed in HEK293 cells. No enzyme activity or protein band of I758D and I758R was found in the medium. Similarly, F756D and F756R, but not F756I, showed no enzyme activity in cell lysates and culture medium. Correspondingly, the 133-kDa and 130-kDa mature forms were not found in the cell lysate and culture medium, respectively. The replacement of Phe756 with Ile had little effect. Next, the cell surface distribution of the enzyme was examined with permeable (permeable condition) or without (unpermeable condition) Triton X-100 treatment. Mutants I758D, I758R, F756D, and F756R, all of which lost enzyme activity completely, were not stained with anti-Myc antibody under unpermeable conditions, indicating they were not expressed on the surface of cells (Fig. 4B, lower panels). However, under permeable conditions these mutant CDases were detected in the ER at almost the same level as the wild-type enzyme (Fig. 4B, upper panels). The cell surface expression of the mutants I758F and F756I is somewhat lower than that of the wild-type and I758V enzymes. This result is well consistent with the enzyme activity levels. No reduction in enzyme activity or cell-surface expression was observed when Glu757, a non-conserved amino acid, was replaced with Arg (control experiment). In summary, these results clearly indicate that the conserved amino acid residues in the COOH-terminal tail, Ile758 and Phe756, are integral to the activity and correct subcellular localization of the enzyme. It is noteworthy that Ile758 or Phe756 can be changed with another hydrophobic, but not a charged, amino acid without a marked loss of enzyme activity.

**Glycosylation of Truncated and Mutated CDases**—We compared the glycosylation of wild-type and mutant CDases using endoglycosidase H (endo H) and glycopeptidase F (PNGF). The former specifically hydrolyzes the N,N'-chitobiose linkage in high-mannose type N-glycans whereas the latter hydrolyzes not only high mannos-type, but also complex and hybrid-type N-glycans. As previously reported (9), the 113-kDa CDase was sensitive to endo H, whereas the 133-kDa CDase was somewhat resistant to endo H (Fig. 5A). The 133-kDa band was converted to a 105-kDa band after PNGF treatment. In contrast, both the I758D and Δ4 CDases showed only an endo H-sensitive 113-kDa band (Fig. 5A), indicating that the mutant enzymes possess high mannos, but not complex/hybrid, type N-glycans. Treatment of the cells overexpressing wild-type CDase with benzyl-GalNAc, an inhibitor of O-glycosylation, resulted in a reduction in the molecular mass of the 133-kDa mature form, but not the 113-kDa ER form (Fig. 5B). In contrast, the I758D and Δ4 CDases were not affected by the inhibitor, indicating that these mutant enzymes are not glycosylated with O-glycans (Fig. 5B). Δ3 CDase seems to enter the ER/Golgi
pathway and so be glycosylated properly, although the processing is somewhat slower than that of the wild-type enzyme (Fig. 5, A and B). Next, we examined the intracellular localization of the wild-type and I758D CDases. The Myc signal of wild-type CDase was partly co-localized with the signal for Rab6, a marker protein for the Golgi apparatus, but that of I758D mutant CDase was not (Fig. 5C). Similarly, Δ4 and F756D CDases were not co-localized with Rab6 (data not shown). These results suggest that the removal of more than 3 amino acids from the COOH terminus or point mutations of two conserved amino acids in the COOH-terminal tail stop the transport of the enzyme from the ER to the Golgi apparatus, resulting in an absence of the protein on plasma membranes (Figs. 3A and 4B). It was concluded that the aberrant COOH-terminal mutant CDases lacking enzyme activity are not transported to the Golgi apparatus leading to the immature/incorrect glycosylation of the protein.

Susceptibility to Degradation by Trypsin—The loss of enzyme activity and incorrect subcellular localization of the aberrant COOH-terminal enzymes could be attributed to misfolding of the proteins. To assess this possibility, the susceptibility of mutant CDases to trypsin was examined since trypsin-sensitivity may reflect the misfolding of proteins (21). As shown in Fig. 6, a notable difference in susceptibility to trypsin was observed between the wild-type and mutant CDases. Both the 133- and 113-kDa protein bands of wild-type CDase were quite resistant to proteolysis by trypsin up to a concentration of 100 ng/μl, the 133-kDa mature form still remaining after digestion for 30 min with 500 ng/μl of trypsin. In contrast, I758D and Δ4 CDases were completely degraded by trypsin at a concentration of 50 ng/μl, being 10-times as sensitive to trypsin as the wild-type enzyme. It is noted that Δ3 CDase was relatively resistant to trypsin compared with I758D and Δ4 CDases. These results may indicate that a mutation or truncation of the COOH-terminal tail leads to incorrect folding of the neutral CDase.

Degradation of COOH-terminal-truncated and -mutated CDases by Proteasome—We examined the effects of cycloheximide (CHX), an inhibitor for de novo protein synthesis, on the stability of CDases overexpressed in HEK293 cells. As shown in Fig. 7A, the 113-kDa form disappeared quickly when de novo synthesis of the protein was stopped by CHX while the 133-kDa mature forms of the wild-type and Δ3 CDases were detected after treatment of the cells with CHX for 4 h. The quick disappearance is probably caused by the conversion of the 113-kDa mature forms of the wild-type and Δ3 CDases into the 133-kDa form. The I758D and Δ4 CDases disappeared very quickly after treatment of the cells with CHX, suggesting that they were degraded much faster than the wild-type enzyme in cells. To examine whether the proteasome is
involved in the quick disappearance of the I758D and Δ4 CDases, cells were incubated with MG-132, a specific inhibitor for the proteasome (22). Treatment with MG-132 resulted in a marked accumulation of I758D and Δ4 CDases in the cell lysates (Fig. 7B). In contrast, MG-132 had no notable effects on the content of wild-type CDase regardless of the attachment of a FLAG tag at the NH2-terminal (Fig. 7B). These results suggest that the I758D and Δ4 CDases, which are likely to be folded incorrectly in the ER, are degraded quickly by the proteasome.

Effects of COOH-terminal Truncation and Mutation on Pseudomonas CDase—To investigate the role of the COOH-terminal tail of bacterial CDases, DNA constructs encoding truncated and point-mutated CDases of Pseudomonas aeruginosa were designed (Fig. 8A) and expressed in E. coli BL21 (DE3)pLysS cells. Val665 to Asp or removal of Val665 completely abrogated the enzyme activity (Fig. 8B). However, Δ5 CDase possessing Val665 retained the enzyme activity though the reaction was slower than for the wild-type CDase (Fig. 8B). The protein expression levels of these three mutant enzymes were almost the same as wild-type levels when determined by Western blotting using anti-Pseudomonas CDase antibody (Fig. 8C). To investigate the susceptibility to proteolysis, wild-type and mutated enzymes were treated with trypsin for 30 min at different concentrations. It was found that the V665D and Δ6 mutants were very sensitive to trypsin compared with the wild-type enzyme (Fig. 8D). Taken together, it is concluded that the

![Image](https://example.com/image.png)
COOH-terminal tail is indispensable for the correct folding and activity of the *Pseudomonas* enzyme.

**DISCUSSION**

Interestingly, the shortened rat neutral CDases differing in length by only one amino acid (Δ4 versus Δ3 enzyme) have quite different fates when overexpressed in HEK293 cells, i.e. Δ4 CDase is not transported from the ER to the Golgi apparatus, instead it is degraded possibly by the proteasome, while Δ3 enzyme is transported normally to the plasma membranes via the Golgi and partly detached from the cells. The reason for the difference was revealed by point mutation analysis: the 4th amino acid residue from the COOH terminus, Ile758, is essential for the correct folding and localization of the enzyme. In addition, Phe756, another conserved amino acid residue at the COOH-terminal tail, was also shown to be very important for enzyme functions. Therefore, two conserved amino acid residues in the COOH-terminal tail are indispensable for the correct folding and localization, and enzyme activity of the neutral CDase. It is noteworthy that these two hydrophobic amino acids could be replaced with other hydrophobic amino acids without a marked loss of function while their replacement with non-hydrophobic amino acids caused critical damage to the enzyme. X-ray crystallography or NMR data is, however, required to reveal how the COOH-terminal tail contributes to the correct folding of the enzyme.

In eukaryotic cells, the ER has the inherent function of ensuring that only correctly folded and assembled proteins are forwarded to their destinations. The term quality control has been adopted to describe the process of conformation-dependent molecular sorting of newly synthesized proteins in the ER (23). Most commonly, misfolded or incompletely assembled proteins are retained in the ER and eventually degraded by the ubiquitin-proteasome pathway (23). The fact that the truncated and mutated neutral CDases were retained in the ER and degraded possibly by the proteasome may indicate that the aberrant COOH-terminal sequence resulted in a misfolding of the protein leading to elimination via quality control mechanisms coupled to the ER.

There have been several reports that COOH-terminal residues are important for the correct folding and secretion of proteins (21, 24–26). Occasionally, COOH-terminal mutations are known to cause genetic disorders (16, 27). In human α-1-proteinase inhibitor deficiency, some variants are not secreted due to frameshift mutations leading to products with aberrant COOH-terminal sequences (28). The low level of the protein results in a failure to inhibit elastase released from activated or disintegrating neutrophils, and causes pulmonary damage in...
emphysema (28). Farber disease is an autosomal recessive disorder caused by a deficiency of acid CDase (29). In one patient with a severe Farber phenotype, there was an insertion of the base T at the stop codon, which caused a frameshift and change in the COOH-terminal structure (16). These reports may indicate that a frameshift mutation at the COOH terminus can lead to a complete loss of function of the neutral CDase although a genetic deficiency of the enzyme has yet to be identified.

This is the first report demonstrating the importance of the COOH-terminal tail of neutral CDase, which would be involved in the regulation of Cer-mediated signaling (11–13), for folding and processing leading to the enzyme activity and distribution. It should be emphasized that the role of the COOH-terminal tail of the enzyme has not changed throughout evolution, because aberrant COOH-terminal sequences drastically affect the functions of not only mammalian but also bacterial enzymes.

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REFERENCES

1. Pettus, B. J., Charlant, C. E., and Hannun, Y. A. (2002) Biochem. Biophys. Acta 1585, 114–125
2. Cuvillier, O. (2002) Biochem. Biophys. Acta 1585, 153–162
3. Spiegel, S., and Kolesnick, R. (2002) J. Biol. Chem. 278, 3131–3133
4. Ito, M., Osato, T., Tani, M., Mitsuoka, S., and Kita, K. (2003) in Ceramide Signaling (Putnam, A. H., ed) pp. 41–48, Landes Bioscience, Georgetown, TX
5. Okino, N., Ichinose, S., Osato, T., Imayama, S., Nakamura, T., and Ito, M. (1999) J. Biol. Chem. 274, 36616–36622
6. Yoshimura, Y., Okino, N., Tani, M., and Ito, M. (2002) J. Biochem. 132, 229–236
7. Sugita, M., Williams, M., Dulaney, J. T., and Moser, H. W. (1975) Biochim. Biophys. Acta 411, 227–238
8. Tani, M., Okino, N., Mori, K., Tanigawa, T., Izu, H., and Ito, M. (2000) J. Biol. Chem. 275, 11229–11234
9. Mitsuoka, S., Tani, M., Okino, N., Mori, K., Ichinose, S., Osato, T., Ida, H., Nakamura, T., and Ito, M. (2001) J. Biol. Chem. 276, 26249–26259
10. El Bawab, S., Reddy, P., Qian, T., Bielawska, A., Lemasters, J. J., and Hannun, Y. A. (2000) J. Biol. Chem. 275, 21508–21513
11. Franzen, R., Pautz, A., Brautigam, L., Geisslinger, G., Pfeilschifter, J., and Huwiler, A. (2001) J. Biol. Chem. 276, 35382–35389
12. Zhang, Z., Mandal, A. K., Mital, A., Popescu, N., Zimonjic, D., Moser, A., Moser, H., and Mukherjee, A. B. (2000) Mol. Genet. Metab. 70, 301–309
13. Tani, M., Iida, H., and Ito, M. (2003) J. Biol. Chem. 278, 10525–10530
14. Okino, N., Iida, H., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373
15. Zhang, Z., Mandal, A. K., Mital, A., Popescu, N., Zimonjic, D., Moser, A., Moser, H., and Mukherjee, A. B. (2000) Mol. Genet. Metab. 70, 301–309
16. Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Anal. Biochem. 263, 183–188
17. Tani, M., Okino, N., Mitsuoka, S., Tanigawa, T., Izu, H., and Ito, M. (2000) J. Biol. Chem. 275, 3462–3468
18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
20. Tsukuba, T., and Bond, J. S. (1998) J. Biol. Chem. 273, 35260–35267
21. Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Anal. Biochem. 263, 183–188
22. Domowicz, M. S., Priok III, E. W., Novak, T. E., and Schwartz, N. B. (2000) J. Biol. Chem. 275, 25098–25105
23. Ellgaard, L., Molinari, M., and Hardingham, T. E. (1999) Science 286, 1882–1888
24. Domowicz, M. S., Priok III, E. W., Novak, T. E., and Schwartz, N. B. (2000) J. Biol. Chem. 275, 35098–35105
25. Lydakis-Simantiras, N., Betts, S. D., and Youm, C. F. (1999) Biochemistry 38, 15528–15535
26. Shimada, Y., Nakamura, M., Naito, Y., Nomura, K., and Ohno-Iwashita, Y. (1999) J. Biol. Chem. 274, 18536–18542
27. Crystal, R. G. (1989) Trends Genet. 5, 411–417
28. Brodbeck, R. M., and Brown, J. L. (1992) J. Biol. Chem. 267, 294–297
29. Spiegel, S., and Kolesnick, R. (2002) J. Biol. Chem. 278, 3131–3133
30. Thompson, J. D., Higgins, D. J., and Gibson, T. J. (1994) Nucleic Acids Res. 22, 4673–4680
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