Ceramidase Enhances Phospholipase C-induced Hemolysis by Pseudomonas aeruginosa*

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We previously reported the purification, molecular cloning, and characterization of a neutral ceramidase from Pseudomonas aeruginosa strain AN17 (Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373; Okino, N., Ichi-nose, S., Omori, A., Imayama, S., Nakamura, T., and Ito, M. (1999) J. Biol. Chem. 274, 36616–36622). Interestingly, the gene encoding the enzyme is adjacent to that encoding hemolytic phospholipase C (plcH) in the genome of Pseudomonas aerugi-nosa, which is a well known pathogen for opportunistic infections. We report here that simultaneous production of PlcH and ceramidase was induced by several lipids and PlcH-induced hemolysis was significantly enhanced by the action of the ceramidase. When the strain was cultured with sphingomyelin or phosphatidylcholine, production of both enzymes drastically increased, causing the increase of hemolytic activity in the cell-free culture supernatant. Ceramidase and sphingosine were also effective in promoting the production of ceramidase but not that of PlcH. Furthermore, we found that the hemolytic activity of a Bacillus cereus sphingomyelinasinase was significantly enhanced by addition of a recombinant Pseudomonas cerami-dase. TLC analysis of the erythrocytes showed that ceramide produced from sphingomyelin by the sphingomyelinasinase was partly converted to sphingosine by the ceramidase. A ceramidase-null mutant strain caused much less hemolysis of sheep erythrocytes than did the wild-type strain. Sphingosine was detected in the erythrocytes co-cultured with the wild-type strain but not the mutant strain. Finally, we found that the enhancement of PlcH-induced hemolysis by the ceramidase occurred in not only sheep but also human erythrocytes. These results may indicate that the ceramidase enhances the PlcH-induced cytotoxicity and provide new insights into the role of sphingolipid-degrading enzymes in the pathogenicity of P. aeruginosa.

Ceramidase (CDase, EC 3.5.1.23) is an enzyme that catalyzes the hydrolysis of the N-acyl linkage of ceramide (Cer) to genera-

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2 The abbreviations used are: CDase, ceramidase; Cer, ceramide; HSA, human serum albumin; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; PC, phosphatidylcholine; PLC, phospholipase C; PY, peptone-yeast extract; RT, reverse

ate sphingosine (Sph) and fatty acid (1). Because Sph is produced not through de novo synthesis but from Cer by the action of CDase (2), CDase is a rate-limiting enzyme in control of the intracellular level of Cer/Sph/sphingosine 1-phosphate and is also crucial for Cer/Sph/sphingosine 1-phosphate-mediated signaling. Based on optimal pH and primary structure, CDases are classified into three groups: acid, neutral, and alkaline enzymes (3). Because we first purified and cloned a neutral CDase from Pseudomonas aeruginosa (4, 5), neutral CDases have been cloned from slime mold (6), Drosophila (7), zebrafish (8), rat (9), mouse (10), and human (11, 12). Interestingly, bacterial CDases cloned from P. aeruginosa, Mycobacterium tuber-culosis (5), and Dermatophilus congolensis (13), are classified as neutral CDases based on primary structure. The functions of neutral CDases have been investigated not only at the cellular level but also at the individual level. Recently, Acharya et al. (14, 15) suggested that neutral CDase is important for the functions of photoreceptors in Drosophila. We also reported that neutral CDase is essential for the metabolism of Cer in zebrafish embryogenesis (8). However, the physiological functions of bacterial CDases are still unknown.

P. aeruginosa is a Gram-negative pathogen capable of causing great morbidity and mortality in burn victims, those with cystic fibrosis, and immunocompromised patients (16). The bacteria have been also found in dermatitic lesions of sheep suffering from fleece rot, which is characterized by superficial inflammation of the skin. Like many other pathogens, P. aerugi-nosa secretes a number of extracellular virulence factors including exotoxins, proteases, hemolysins, and phospho-lipases (17). Two hemolysins produced by the pathogen are a heat-stable glycolipid (18) and a heat-labile phospholipase C (PLC) known as the hemolytic PLC (PlcH) (19). Furthermore, P. aeruginosa elaborates two homologous extracellular PLCs, a PlcH and a non-hemolytic PLC (PlcN) (20). Although PlcH and PlcN show ~40% identity at the amino acid level, substrate specificity of these two PLCs are quite different, PlcH hydrolyzes phosphatidylcholine (PC), lyso-PC, and sphingomyelin (SM), whereas PlcN hydrolyzes PC and phosphatidylserine but not lyso-PC or SM (20, 21). Very recently, a novel extracellular PLC (PclcB) was cloned using a bioinformatics approach (22). Disruption of the pclcB gene revealed that PlcB is essential for directed twitching motility up a phospholipid gradient of certain species of either phosphatidylethanolamine or PC (22).
In this paper, we report the role of a bacterial CDase in *P. aeruginosa* for the first time. Our study clearly indicates that the CDase enhances PlcH-induced hemolysis of sheep as well as human erythrocytes. This observation would explain, in part, how PlcH of *P. aeruginosa* is toxic to host cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—*N*-Palmitoylsphingosine, SM, *d*-erythro-Sph, sodium taurodeoxycholate, C6-NBD-SM, fatty acid-free albumin from human serum (HSA), and Triton X-100 were purchased from Sigma. Anti-*Pseudomonas* CDase antibody was raised in a rabbit using recombinant *Pseudomonas* CDase as the antigen (23). SMase from *Bacillus cereus* was obtained from Funakoshi (Tokyo, Japan). Horseradish peroxidase-labeled anti-rabbit IgG antibody was purchased from Nakalai Tesque (Kyoto, Japan). C12-NBD-Cer was prepared by a method reported previously (24). A precoated Silica Gel 60 TLC plate was purchased from Merck (Darmstadt, Germany). The pK19mobsacB vector (25) was donated by Dr. S. Yasuda, National Institute of Genetics, Japan. CDase and PlcH from *P. aeruginosa* were cloned and expressed in *Escherichia coli* as a polyhistidine-tagged protein at the C terminus. The expressed proteins were purified by Ni-Sepharose 6 Fast Flow (Amersham Biosciences) according to the manufacturer’s instructions. All other reagents were of the highest purity available.

**Bacterial Strains and Media**—A type strain of *P. aeruginosa* IFO12689 was obtained from the Institute for Fermentation, Osaka (IFO), Japan. *P. aeruginosa* strains were cultivated in PY-medium (0.5% polypeptone, 0.1% yeast extract, and 0.5% NaCl, pH 7.2) at 30 °C. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. The media were supplemented with antibiotics such as ampicillin (100 μg/ml for *E. coli*), carbenicillin (100 μg/ml for *E. coli*), kanamycin (50 μg/ml for *E. coli*), and tetracycline (15 μg/ml for *E. coli* and 200 μg/ml for *P. aeruginosa*) when necessary.

**Preparation of the Culture Supernatant**—Inocula from an agar plate of *P. aeruginosa* strain AN17 were introduced into a 14-ml test tube containing 2 ml of sterilized PY-medium, and incubated at 30 °C for 1 day with vigorous shaking. Cells of the AN17 strain were harvested by centrifugation at 11,000 × g for 1 min, washed once with 1 ml of saline, and resuspended in saline (A<sub>600 nm</sub> = 2.0). A 10-μl aliquot of the cell suspension was inoculated into 2 ml of PY-medium containing 0.05% sodium taurodeoxycholate and 100 μM of each lipid, and shaken at 30 °C for 24 h. The culture fluid was centrifuged at 11,000 × g for 1 min, and the supernatant obtained was used for the enzyme assay.

**SDS-PAGE and Western Blotting**—SDS-PAGE was carried out according to the method of Laemmli (26). Protein transfer onto a polyvinylidene difluoride membrane was performed using TransBlot SD (Bio-Rad) according to the instructions of the manufacturer. After treatment with 5% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (T-TBS) for 1 h, the membrane was incubated at 4 °C overnight with anti-*Pseudomonas* CDase antibody. After a wash with T-TBS, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody for 2 h. After another wash with T-TBS, the ECL reaction was performed for 10 min as recommended by the manufacturer, and chemiluminescence was detected with an ECL Mini-camera (Amersham Biosciences).

**Assay of CDase**—The activity of CDase was measured using C12-NBD-Cer as a substrate as described below. The reaction mixture contained 1 nmol of C12-NBD-Cer and an appropriate amount of the enzyme in 20 μl of 25 mM Tris-HCl buffer, pH 8.5, containing 0.25% (w/v) Triton X-100 and 2.5 mM CaCl<sub>2</sub>. Following incubation at 37 °C for an appropriate time, the reaction was terminated by adding 50 μl of chloroform/methanol (2/1, v/v), mixed, and then centrifuged at 16,000 × g for 1 min. Five microliters of the upper phase were applied to a TLC plate, which was developed with chloroform, methanol, 25% ammonia (14/6/1, v/v) as a developing solvent. The C12-NBD-fatty acid released by the action of the enzyme and the remaining C12-NBD-Cer were then analyzed and quantified with a Shimadzu CS-9300 chromatograph (Shimadzu, Kyoto, Japan) (excitation, 475 nm; emission, 525 nm). One enzyme unit of CDase was defined as the amount capable of catalyzing the release of 1 μmol of C12-NBD-fatty acid/min from the C12-NBD-Cer under the conditions described above. A value of 10<sup>−3</sup> unit enzyme was expressed as 1 milliunit in this study.

**Assay of SMase**—The activity of SMase was measured using C6-NBD-SM as a substrate as described below. The reaction mixture contained 1 nmol of C6-NBD-SM and an appropriate amount of the enzyme in 20 μl of 25 mM Tris-HCl buffer, pH 7.5, containing 0.1% (w/v) Triton X-100. Assays were generally carried out at 37 °C for an appropriate time. The reactions were terminated by adding 50 μl of chloroform/methanol (2/1, v/v), and the reaction mixture was then centrifuged at 16,000 × g for 1 min. The C6-NBD-SM released by the action of the enzyme and the remaining C6-NBD-SM were analyzed and quantified as described above. One enzyme unit of SMase was defined as the amount capable of catalyzing the release of 1 μmol of C6-NBD-SM/min from the C6-NBD-SM under the conditions described above. A value of 10<sup>−3</sup> units of enzyme was expressed as 1 milliunit in this study.

**Assay of Hemolysis**—The hemolytic activity was examined by adding the sample to a 10% sheep erythrocyte suspension in TBS, followed by incubation at 37 °C for 60 min. After the reaction, the reaction mixture was centrifuged at 1,000 × g for 1 min. The supernatant was then diluted with distilled water (3 volumes) and the release of hemoglobin was measured using a spectrophotometer at 490 nm.

**Reverse Transcription-PCR (RT-PCR)**—For RT-PCR experiments, cells were grown in PY-medium as described above for 9 h (late exponential phase). Total cellular RNA was isolated from the cells using a Qiagen RNeasy Mini Kit (Qiagen Inc.), treated with an RNase-free DNase Set (Qiagen), and re-purified using the same kit. DNase-treated RNA was used as a template for RT-PCR with the Qiagen OneStep RT-PCR kit (Qiagen) according to the manufacturer’s directions. Primers used for the *cdase* were GGCTGGAGGGAGGCAACAAATC (sense) and GCGTAGCCATTGAAGACCAACA (antisense); and for the *plcH*, TCTACGGCCAGTGCTCCAAT (sense) and GGTC-CGGCAGTTCTCTCC (antisense). A 10-μl volume of each reaction mixture was analyzed by agarose (2% w/v) gel electrophoresis to detect the RT-PCR products (for *cdase*, 298 bp; for *plcH*, 299 bp). For quantification of the signals, agarose gel...
gels were stained with ethidium bromide and analyzed with a CS Analyzer (ATTO Corp., Tokyo, Japan).

**Lipid Extraction and TLC Analysis**—For extraction of lipids, the harvested erythrocytes were suspended in 1-propanol/hexane/water (55:35:10, v/v), subjected to sonication for 5 min, and centrifuged at 11,000 × g for 5 min. The supernatants obtained were dried by SpeedVac concentrator, dissolved in 20 μl of chloroform/methanol (2:1, v/v), and then applied to TLC plates, which were developed with chloroform, methanol, 0.02% CaCl2 (5:4:1, v/v) for SM, chloroform/methanol/water (65:25:4, v/v) for Sph, and chloroform, methanol, 25% ammonia (90:10:1, v/v) for Cer. TLC plates were visualized with Coomassie Brilliant Blue R-250 for SM and Cer, and ninhydrin reagent for Sph.

**Measurement of SM Content**—The amount of SM was measured according to the method of He et al. (27). Briefly, erythrocytes were mixed with a 20-fold volume of 0.2% Triton X-100, lysed by vortexing and sonication, and centrifuged briefly in a microcentrifuge. The supernatant obtained was used for the determination of SM content. Five microliters of sample was mixed with 0.4 ml each of chloroform and water, and the chloroform phase was washed with water and dried in a SpeedVac concentrator. The extract was dissolved in 20 μl of ethanol and incubated at 67 °C for 25 min, and mixed with 15 μl of o-phthalaldehyde reaction buffer (9.9 ml of 3% boric acid, pH 10.5, mixed with 0.1 ml of ethanol containing 10 mg of o-phthalaldehyde and 20 μl of β-mercaptoethanol). After incubation at room temperature for 5 min, the sample was then applied to an Inertisol ODS-3 column (4.6 × 100 mm; GL Science Inc.), which was equilibrated with methanol and 5 mM potassium phosphate buffer, pH 7.0, (9/1, v/v).

**Construction of CDase-null Mutant**—A CDase-deficient strain was constructed by insertion of the tetracycline resistance (Tc') cassette into the cdase gene via homologous recombination (25). Because the cdase gene has a unique SacII site at its center, the Tc' cassette of pBR322 was amplified by PCR using a sense primer with a SacII restriction site and an antisense primer with a SacII restriction site (5'-GCCCGCGGT-TCCATTCAGGTCAAGGCG-3'). The resulting 1.2-kb PCR product was digested with SacII and cloned into the SacII site of the full-length cdase gene containing vector pTCD57, generating pTCD57T. The cdase::Tc' construct in pTCD57T was amplified by PCR using a sense primer with an EcoRI restriction site and an antisense primer with an EcoRI restriction site (5'-GGAAATTCGTACGTTCCGCATTCACC-3') and antisense primer with an EcoRI restriction site (5'-AGAATTCCTAGGGAGTGGTGCCGAGCAC-3'). The resulting 3.2-kb PCR product was digested with EcoRI and cloned into pK19mobasC cut with the same enzyme, generating pK19CDT. The plasmid, which contained a mutant cdase disrupted with the Tc' gene, was transferred from the broad host-range mobilizing strain *E. coli* S17-1 (29) to *P. aeruginosa* by biparental filter matings. Tc' plasmid integrants were selected on NAC medium containing Tc. Tc' colonies were then plated on LB agar containing 5% sucrose to identify strains that had lost the vector-associated sacB gene (resistant to sucrose). Gene replacement was ascertained by PCR of the cdase gene.

**RESULTS**

**Induction of CDase, SMase, and Hemolytic Activities by Lipids**—Analysis of the genomic sequence of *P. aeruginosa* PA01 (30) has revealed that the cdase gene (mapped as PA0845) and the hemolytic PLC (plcH) gene (mapped as PA0844) neighbor each other (Fig. 1). To understand how these genes are regulated, we first examined the effect of SM on the growth of strain AN17 and the induction of CDase and PlcH activities. During the exponential phase, SM had no effect on the growth of the strain, however, it significantly enhanced cell growth from the late exponential phase to stationary phase (Fig. 2A). We next analyzed the CDase and PlcH activities in the growth phase. We used C6-NBD-SM for measuring PlcH activity because PlcH hydrolyzes PC as well as SM. Both CDase and PlcH activities were significantly induced in the presence of SM.
The Functional Role of Pseudomonas Ceramidase

Values are expressed as the mean ± S.D. (n = 3). Significance is shown as follows: *, p < 0.001; **, p < 0.005; ***, p < 0.02; ****, p < 0.1 compared with the absence of SM. D, lipid analysis of the culture supernatant. Five hundred microliters of the culture supernatant were dried by a SpeedVac concentrator, dissolved in 30 µl of chloroform/methanol (2:1, v/v), and then applied to TLC plates, which were developed with chloroform, methanol, 0.02% CaCl2 (5:4:1, v/v) for SM, and chloroform/methanol/water (65:25:4, v/v) for Sph and Cer. TLC plates were visualized with Coomassie Brilliant Blue R-250 for SM and Cer, and ninhydrin reagent for Sph. E and F, RT-PCR analysis of cdase and plcH in the presence and absence of SM. P. aeruginosa strain AN17 was grown in PY-medium containing SM for 9 h. RNA was isolated from cells and RT-PCR was performed by the method described under "Experimental Procedures." A 10-µl aliquot of each RT-PCR product was analyzed by 2% (w/v) agarose gel electrophoresis (E) and the signal intensity quantified (F). Values are expressed as the mean ± S.D. (n = 3). Significance is shown as follows: *, p < 0.02; **, p > 0.25 compared with the absence of SM.

(Fig. 2, B and C). The metabolism of the SM added was also analyzed by TLC. As shown in Fig. 2D, in the culture medium, we first detected only SM, which was degraded to Cer and then Sph in a time-dependent manner. After 24 h, no lipids were detected in the culture medium suggesting that these substrates were completely metabolized. Next, the induction of these two enzymes was analyzed at the mRNA level by semi-quantitative RT-PCR. Total RNA was extracted from the cells at a late exponential phase of cell growth (9 h) and RT-PCR was performed using specific oligonucleotide primers to determine the mRNA levels of cdase and plcH. Interestingly, gene expression of cdase was significantly induced by SM, however, that of plcH was not under the conditions used in this study (Fig. 2, E and F). Next, we examined the effect of sphingolipids and their metabolites, and other phospholipids on the production of these two enzymes. When the strain was cultured in the PY-medium containing SM, production of CDase increased significantly (~50-fold) (Fig. 3A). Cer, C2-Cer, Sph, and PC also promoted production of the enzyme (10~20-fold activation), but not as effectively as SM, whereas palmitic acid, phosphatidic acid, diacylglycerol, and phosphatidylserine were ineffective. We next analyzed PlcH activity in the culture medium. When the strain was cultured in the PY-medium containing SM or PC, the secretion of PlcH into the medium increased significantly (~2-fold) (Fig. 3B). However, Cer, C2-Cer, Sph, palmitic acid, and diacylglycerol had almost no effect on the enzymatic activity. Even in the PY-medium without lipids, the strain produced a certain amount of PlcH. This observation is well consistent with the report that PlcH activity was produced under low-phosphate conditions without inducers (31). Phosphatidic acid and phosphatidlyserine inhibited the production of the enzyme, although these lipids did not inhibit the growth of the strain. PlcH has been shown to be a major hemolytic factor of P. aeruginosa (19), and thus we examined the hemolytic activity of the culture supernatant using sheep erythrocytes. As shown in Fig. 3C, the hemolytic activity of this strain is well consistent with the PlcH activity when measured with C6-NBD-SM. Interestingly, hemolytic activity seems to be enhanced in proportion to the production of CDase by this strain, suggesting that CDase

FIGURE 2. Expression of CDase and SMase (PlcH) throughout the growth phase. A, growth curves of P. aeruginosa strain AN17. The strain was grown at 30 °C in 2 ml of PY-medium in the absence or presence of 100 µM SM with shaking. Samples were taken at different times, and the A600 was determined. CDase (B) and SMase (PlcH) (C) activity of the culture supernatant was also measured as described under “Experimental Procedures.” Values are expressed as the mean ± S.D. (n = 3). ■, in the absence of SM; ●, in the presence of 100 µM SM. Significance is shown as follows: *, p < 0.001; **, p < 0.005; ***, p < 0.02; ****, p < 0.1 compared with the absence of SM. D, lipid analysis of the culture supernatant. Five hundred microliters of the culture supernatant of P. aeruginosa strain AN17 was grown in PY-medium containing SM was mixed with 1 ml of chloroform/methanol (2:1, v/v), and centrifuged at 11,000 × g for 5 min. The lower phases were dried by a SpeedVac concentrator, dissolved in 30 µl of chloroform/methanol (2:1, v/v), and then applied to TLC plates, which were developed with chloroform, methanol, 0.02% CaCl2 (5:4:1, v/v) for SM, and chloroform/methanol/water (65:25:4, v/v) for Sph and Cer. TLC plates were visualized with Coomassie Brilliant Blue R-250 for SM and Cer, and ninhydrin reagent for Sph. E and F, RT-PCR analysis of cdase and plcH in the presence and absence of SM. P. aeruginosa strain AN17 was grown in PY-medium in the absence (Cont) or presence (SM) of 100 µM SM for 9 h. RNA was isolated from cells and RT-PCR was performed by the method described under “Experimental Procedures.” A 10-µl aliquot of each RT-PCR product was analyzed by 2% (w/v) agarose gel electrophoresis (E) and the signal intensity quantified (F). Values are expressed as the mean ± S.D. (n = 3). Significance is shown as follows: *, p < 0.02; **, p > 0.25 compared with the absence of SM.

Treatment of Sheep Erythrocytes with Bacterial SMase and CDase—To elucidate the effect of CDase in PlcH-induced hemolysis, sheep erythrocytes were treated with B. cereus SMase, recombinant Pseudomonas CDase, or both. Because sheep erythrocytes are rich in SM but almost lack PC (32), we used a commercially available B. cereus SMase instead of P. aeruginosa PlcH. As shown in Fig. 4A, SMase treatment caused hemolysis of the erythrocytes, and interestingly, simultaneous treatment with SMase and CDase significantly enhanced the hemolysis. This enhancement seems to be due to the action of the CDase, because boiling of the enzyme completely abolished it. However, CDase alone did not cause hemolysis. To disclose the role of CDase in the hemolysis, we analyzed the lipid contents of the erythrocytes with and without treatments with enzymes. As shown in Fig. 4B, Cer was clearly detected in eryth-
rocytes after SMase treatment and a significant amount of Sph was detected in erythrocytes treated simultaneously with SMase and CDase, concomitantly with the decrease in the amount of Cer. It should be noted that boiled CDase did not generate Sph. These results clearly indicate that CDase hydrolyzed Cer, which was generated from SM by the action of SMase and suggest that the conversion of Cer to Sph enhanced the hemolysis.

Disruption of the cdase Gene—Next, we disrupted the cdase gene in P. aeruginosa through insertion of the Tcr gene using a sacB-based gene replacement strategy with the AN17 and IFO12689 strains (authentic P. aeruginosa strain). Disruption of the cdase gene was confirmed by PCR (data not shown) and measuring the CDase activity. The CDase activity of the wild-type and CDase-deficient mutant was measured using C12-NBD-Cer as a substrate when they were cultured in PY-medium containing SM. For the control, the SMase activity of these strains was also measured using C6-NBD-SM. As shown in Fig. 5A, both CDase-deficient mutants lacked any CDase activity, whereas SMase activity was almost the same as for the wild-type strains. Disruption of the cdase gene was also confirmed by Western blotting of the gene product in the culture supernatants using anti-Pseudomonas CDase antibody (Fig. 5B).

Hemolytic Activity of the CDase-null Mutants—Wild-type strains and the CDase-null mutant strains were cultured in PY-medium containing SM and the culture supernatants obtained were subjected to hemolytic assays using sheep, rabbit, and horse erythrocytes. As shown in Fig. 6A, when the culture supernatants were mixed with sheep erythrocytes, the hemolytic activity by the CDase mutants was significantly reduced compared with that by the wild-type (open bar). However, when rabbit erythrocytes were used, the effect of CDase on the hemolysis was very weak (gray bar), i.e. a slight but significant enhancement of hemolysis by the culture supernatant of IFO12689 compared with that of the CDase mutant strain. On the other hand, no significant difference in hemolysis was observed for the culture supernatants of wild-type and the
induced by culturing with sheep erythrocytes. After incubation for 6 and 8 h, significantly less hemolysis was caused by the CDase-null mutant strains than wild-type strains (Fig. 7A, closed square and closed circle versus closed triangle, respectively). To confirm the effect of the CDase on erythrocytes, lipids were extracted from erythrocytes and analyzed by TLC. As shown in Fig. 7B, the time courses for the hydrolysis of SM and hemolysis almost parallel each other. After incubation for 6 h, we detected a significant amount of Sph concomitantly with the reduction in the amount of Cer in the erythrocytes treated with wild-type strains but not the CDase-null mutant strains, indicating that Cer generated on the surface of the erythrocytes by SMase was converted to Sph by CDase, and both enzymes were secreted into the medium. Taken together, it was concluded that CDase enhances PlcH-induced hemolysis caused by P. aeruginosa.

**Hemolysis of Human Erythrocytes**—Finally, we examined the effects of the CDase on human erythrocytes, because P. aeruginosa is an opportunistic pathogen in humans as well as sheep. Similar to the results obtained using sheep erythrocytes (Fig. 6A, open bar), the hemolysis caused by the culture supernatants of the CDase-null mutant strains was much less severe than that caused by the supernatants of the wild-type strains (Fig. 8A). SM and Sph content of the erythrocytes were also analyzed. As shown in Fig. 8, B and C, almost 80% of the SM was hydrolyzed. Importantly, the content of Sph was greatly increased by treatment with the supernatants of the wild-type but not the CDase-null mutant strains. Because HSA is present in blood as a major protein, we examined the effect of HSA on SMase- and SMase-CDase-induced hemolysis. In the presence of HSA, the effect of CDase on SMase-induced hemolysis was greatly enhanced in a concentration-dependent manner (Fig. 9A). To understand how HSA enhances SMase-induced hemolysis, we measured the content of Sph in erythrocytes. As shown in Fig. 9B, a significant amount of Sph was detected in the supernatant when erythrocytes were precipitated by centrifugation, indicating that HSA withdrew Sph from erythrocytes. Interestingly, the content of Sph in CDase-treated erythrocytes also increased in the presence of HSA (Fig. 9C). To exclude the possibility that hemolytic enhancement by HSA is caused by nonspecific protein effects, we performed the same experiment using human apo-transferrin and ovalbumin. The addition of these proteins did not affect CDase-induced hemolytic enhancement (data not shown). Collectively, it was elucidated that HSA enhances SMase-CDase-induced hemolysis by withdrawing Sph from

Co-culture with Bacteria and Erythrocytes—To determine whether the hemolytic enhancement is caused by not only CDase but also the CDase-producing pathogens, sheep erythrocytes were co-cultured with wild-type and CDase-null mutant strains of P. aeruginosa. As shown in Fig. 7A, hemolysis occurred in a time-dependent manner in the co-culture, suggesting that production of PlcH and CDase by bacteria was
CDase-induced hemolysis but also PlcH-induced hemolysis. Furthermore, in the presence of HSA, not only PlcH-induced hemolysis (Fig. 10A). As a result, it was found that CDase significantly enhanced PlcH-induced hemolysis either in the presence or absence of HSA. This result is somewhat different from that of the experiment using SMase instead of PlcH. Furthermore, in the presence of HSA, not only PlcH-CDase-induced hemolysis but also PlcH-induced hemolysis was enhanced. As shown in Fig. 10, B and C, the amount of Sph was greatly increased in the supernatant and cells after treatment of erythrocytes simultaneously with PlcH and CDase in comparison with PlcH only. Collectively, it was revealed that an excess amount of Sph generated by bacterial CDase enhanced both SMase-induced and PlcH-induced hemolysis. Furthermore, we speculate that bacterial CDase could enhance the SMase- or PlcH-induced cytotoxicity for host cells. Because we observed that the growth of WI-38 human lung fibroblasts was completely inhibited by treatment of cells with SMase in the presence of CDase.3 These results suggest that CDase is involved in the PlcH-induced cytotoxicity of P. aeruginosa in humans.

**DISCUSSION**

In this report, we describe the possible functions of the CDase of P. aeruginosa, a famous opportunistic pathogen. The complete genome sequence of P. aeruginosa was reported in 2000 (30), and the gene encoding the neutral cdase was found to lie immediately 5′ upstream of the plcH gene. Because PlcH has been identified as a pathogenic factor in P. aeruginosa (17), we focused on the transcriptional regulation of these two genes. We first examined the induction of the enzymes by various sphingolipids and their metabolites, and other phospholipids. The amount of CDase secreted into the culture medium

was greatly increased in the supernatant and cells after treatment of erythrocytes simultaneously with PlcH and CDase, in comparison with PlcH only. Collectively, it was revealed that an excess amount of Sph generated by bacterial CDase enhanced both SMase-induced and PlcH-induced hemolysis. Further-

more, we speculate that bacterial CDase could enhance the SMase- or PlcH-induced cytotoxicity for host cells. Because we observed that the growth of WI-38 human lung fibroblasts was significantly inhibited by treatment of cells with SMase in the presence of CDase while no inhibition occurred by treatment with SMase in the absence of CDase.3 These results suggest that CDase is involved in the PlcH-induced cytotoxicity of P. aeruginosa in humans.

**FIGURE 7. Hemolytic activity of wild-type and the CDase-null mutant of P. aeruginosa.** A, time course for hemolysis of sheep erythrocytes by wild-type and the CDase-null mutant of P. aeruginosa. Fifty microliters of the cell suspension (A400 = 0.4) in TBS was mixed with 50 μl of the erythrocytes in TBS and incubated at 30 °C for the period indicated. After incubation, the reaction mixtures were centrifuged at 1,000 g for 1 min and the supernatants were analyzed for hemolytic activity by the method described under “Experimental Procedures.” Values are expressed as the mean ± S.D. (n = 3). CONT: control supernatant; AN17: AN17; AN17 ΔCD; AN17 ΔCD.

**FIGURE 6. Hemolytic activity of culture supernatants from wild-type and the CDase-null mutant of P. aeruginosa.** A, hemolytic activity of culture supernatants. Wild-type and the CDase-null mutant of P. aeruginosa were cultured for 24 h at 30 °C in PY-medium containing 100 μM SM. The culture supernatants were subjected to SM analysis by the method described under “Experimental Procedures.” Values are expressed as the mean ± S.D. (n = 3). TX-100, Triton X-100.
was very small in the absence of lipids, but increased significantly in the presence of SM. Cer, C2-Cer, Sph, and PC were also effective in inducing production of the enzyme. In contrast, only SM and PC were found to induce the production of SMase (PlcH) among the lipids tested, but a certain amount of the enzyme was constitutively produced in the absence of lipids. This is well consistent with the report that *plcH* is a phosphate-regulated gene, which is expressed under low concentrations of phosphate (31). Furthermore, RT-PCR analysis of *cdase* and *plcH* genes revealed that these two genes were expressed in the late exponential phase and at least the *cdase* gene expression is significantly up-regulated by SM. These results clearly indicate that *P. aeruginosa* secretes both SMase and CDase in the presence of SM and PC, which are usually abundant in plasma membranes of mammalian cells, and suggest that the induction of these enzymes is regulated by a somewhat different mechanism.
The Functional Role of Pseudomonas Ceramidase

Surprisingly, during the analysis of the hemolytic activity, it was found that CDase enhanced the hemolysis caused by PlcH. We therefore examined how CDase enhances PlcH-induced hemolysis. Treatment of sheep erythrocytes simultaneously with B. cereus SMase and Pseudomonas CDase caused an increase in hemolysis compared with SMase treatment only. Furthermore, we detected Sph in the erythrocytes treated with SMase and CDase but not SMase or CDase only, indicating that cell surface SM was first hydrolyzed to generate Cer by the SMase, and then Cer was further hydrolyzed to Sph by the CDase. The hydrolysis of SM and PC on the outer leaflet of the plasma membrane by PLC and SMase may cause damage to the membrane, which leads to lysis of the erythrocytes (33). Colley et al. (34) reported that treatment of human erythrocytes with B. cereus SMase or PLC \textit{per se} was not able to induce hemolysis, but the combination of these two enzymes induced complete hemolysis. They also reported that SMase but not PLC induced partial hemolysis in the presence of fatty acid-free bovine serum albumin. In the present study we found that HSA enhanced not only SMase- or PlcH-induced hemolysis but also SMase-CDase-induced or PlcH-CDase-induced hemolysis. Although how CDase enhances SMase- or PlcH-induced hemolysis in the presence of HSA is still unclear at present, it was elucidated that the conversion of Cer to Sph followed by withdrawal of Sph from the plasma membrane by HSA accelerated hemolysis. It is worth noting that lysosphingolipids but not parental sphingolipids, can disrupt erythrocyte membranes via a nonspecific detergent-like effect (35–37). Sph, a kind of lysosphingolipid, may have a similar effect.

Several lines of evidence suggest that hydrolysis of not only PC but also SM is important for bacterial PLC-induced hemolysis. Bacterial PLCs, which hydrolyze both PC and SM, are important virulence factors in the diseases caused by \textit{Listeria monocytogenes}, \textit{Clostridium perfringens}, and \textit{P. aeruginosa} (33, 38). In addition, \textit{M. tuberculosis} possesses four functional PLC genes, two of which encode proteins having SMase activity. Disruption of these genes revealed that these PLCs are involved in the virulence of \textit{M. tuberculosis} (39). Interestingly, \textit{M. tuberculosis} also has a functional \textit{cdase} gene in its genome. Because the \textit{Mycobacterium} CDase does not have a signal sequence for secretion, it is likely to have a different function to the \textit{Pseudomonas} CDase. In experiments using \textit{P. aeruginosa}, PlcH played a role in pathogenesis (40–42), i.e. the PlcH mutant exhibited less virulence in a mouse burn model. Furthermore, patients with chronic infections of \textit{P. aeruginosa} and sheep with fleece rot show high titers for antibody against PlcH (43, 44), indicating that PlcH is produced \textit{in vivo} under certain conditions. In this study, we revealed that production of both CDase and PlcH is induced by SM and PC, which are abundant in the outer leaflet of plasma membranes. Furthermore, the co-culture of sheep erythrocytes with \textit{P. aeruginosa} induced expression of SMase as well as CDase. These results suggest that infections of \textit{P. aeruginosa} induce production of not only SMase but also CDase \textit{in vivo} and the CDase may increase the SMase-induced cytotoxicity.

Many pathogenic and opportunistic microbes produce SMase causing the generation of Cer in hosts. Because Cer is toxic to host cells, it should be excluded by the defense system of the host. For example, Komori et al. (45, 46) reported that Cer generated in the outer leaflet of the plasma membrane by bacterial SMase was metabolized to glycosphingolipids in B16 melanoma cells through activation of a glucosylceramide synthase. Recently, Huitema et al. (47) reported the cloning of human SM syntheses (SMS1 and SMS2) and demonstrated that SMS1 is located in the endoplasmic reticulum, whereas SMS2 is present in the plasma membrane. Thus, SMS2 may also be
The Functional Role of Pseudomonas Ceramidase

Bacteroides, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) Rev. Infect. Dis. 5, 279–313

Vasili, M. L. (1986) J. Pediatr. 108, 800–805

Fujita, K., Akino, T., and Yoshioka, H. (1988) Infect. Immun. 56, 1385–1387

Vasili, M. L., Berka, R. M., Gray, G. L., and Nakai, H. (1982) J. Bacteriol. 152, 431–440

Ostroff, R. M., Vasili, A. I., and Vasili, M. L. (1990) J. Bacteriol. 172, 5915–5923

Stonehouse, M. J., Cota-Gomez, A., Parker, S. K., Martin, W. E., Hankan, J. A., Murphy, R. C., Chen, W., Lim, K. B., Hackett, M., Vasili, A. I., and Vasili, M. L. (2002) Mol. Microbiol. 46, 661–676

Barker, A. P., Vasili, A. I., Filloux, A., Ball, G., Wilderman, P. I., and Vasili, M. L. (2004) Mol. Microbiol. 53, 1089–1098

Tani, M., Okino, N., Sueyoshi, N., and Ito, M. (2004) J. Biol. Chem. 279, 29351–29358

Tani, M., Kita, K., Komori, H., Nakagawa, T., and Ito, M. (1998) Anal. Biochem. 263, 183–188

Schäfer, A., Tauc, A., Jäger, W., Kelinowski, J., Thierbach, G., and Pühlér, A. (1994) Angew. Chem. 145, 69–73

Laemmli, U. K. (1970) Nature 227, 680–685

He, X., Chen, F., McGovern, M. M., and Schuchman, E. H. (2002) Anal. Biochem. 306, 115–123

Monjusho, H., Okino, N., Tani, M., Maeda, M., Yoshida, M., and Ito, M. (1999) J. Biol. Chem. 274, 2385–2393

Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., and Sandhoff, K. (1999) J. Biol. Chem. 274, 2385–2393

Futerman, A. H. (ed) pp. 41–48, Landes Bioscience, Georgetown, TX

Kawamura, N. (1970) J. Biol. Chem. 245, 331–337

Takemoto, T., and Kawamura, N. (1970) J. Biol. Chem. 245, 331–337

Pritchard, A. E., and Vasil, M. L. (1986) J. Biol. Chem. 261, 291–298

Nelson, G. J. (1967) Biol. Chem. Acta 144, 221–232

Tibball, R. W. (1993) Microbiol. Rev. 57, 347–366

Colley, C. M., Zwaal, R. F., Roelofs, B., and van Deenen, L. L. (1973) Biochim. Biophys. Acta 119, 37–42

Nakagawa, T., Morotomi, A., Tani, M., Sueyoshi, N., Komori, H., and Ito, M. (2005) J. Lipid Res. 46, 1103–1112

Songer, J. G. (1997) Trends Microbiol. 5, 156–161

Raynaud, C., Guilhot, C., Rauzier, J., Bordat, Y., Pelicic, V., Manganelli, R., Smith, I., Gicquel, B., and Jackson, M. (2002) Mol. Microbiol. 45, 203–217

Ostroff, R. M., Wretlind, B., and Vasili, M. L. (1989) Infect. Immun. 57, 1369–1373

Meyers, D. J., Palmer, K. C., Bale, L. A., Kernacki, K., Preston, M., Brown, T., and Berk, R. S. (1992) Toxicon 30, 161–169

Rehme, L. G., Ausubel, F. M., Cao, H., Drenkard, E., Goumnerov, B. C., Garber, R. L., Goltry, L., Tolentino, E., Westbrook-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., Paulsen, I. T., Reizer, J., Saier, M. H., Hancock, R. E., Lory, S., and Olson, M. V. (2000) Nature 406, 959–964

Chin, J. C., and Watts, J. E. (1988) J. Gen. Microbiol. 134, 2567–2575

Komori, H., Ichikawa, S., Hirabayashi, Y., and Ito, M. (1999) J. Biol. Chem. 274, 9891–9897

Huijtena, K., van den Dijken, J., Brouwers, J. F., and Holthuis, J. C. (2004) EMBO J. 14, 33–44

Tani, M., Igarashi, Y., and Ito, M. (2005) J. Biol. Chem. 280, 36592–36600

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REFERENCES

1. Gatt, S. (1963) J. Biol. Chem. 238, 3131–3133

2. Michel, C., van Echten-Deckert, G., Rother, J., Sandhoff, K., Wang, E., and Merrill, A. H., Jr. (1997) J. Biol. Chem. 272, 22432–22437

3. Ito, M., Okino, N., Tani, M., Mitsutake, S., and Kita, K. (2003) in Ceramidase Signalng (Puterman, A. H., ed) pp. 41–48, Landes Bioscience, Georgetown, TX

4. Okino, N., Tani, M., Imayama, S., and Ito, M. (1998) J. Biol. Chem. 273, 14368–14373

5. Okino, N., Ichinoso, S., Omori, A., Imayama, S., Nakamura, T., and Ito, M. (1999) J. Biol. Chem. 274, 36616–36622

6. Monjusho, H., Okino, N., Tani, M., Maeda, M., Yoshida, M., and Ito, M. (2003) Biochem. J. 376, 473–479

7. Yoshimura, Y., Okino, N., Tani, M., and Ito, M. (2002) J. Biochem. (Tokyo) 132, 229–236

8. Yoshimura, Y., Tani, M., Okino, N., Iida, H., and Ito, M. (2004) J. Biol. Chem. 279, 44012–44022

9. Mitsutake, S., Tani, M., Okino, N., Mori, K., Ichinoso, S., Omori, A., Iida, H., Nakamura, T., and Ito, M. (2001) J. Biol. Chem. 276, 26249–26259

10. Tani, M., Okino, N., Mori, K., Tanigawa, T., Iza, H., and Ito, M. (2000) J. Biol. Chem. 275, 11229–11234

11. Bawab, S. E., Robby, P., Qian, T., Bielewska, A., Lemasters, J. I., and Hanun, Y. H. (2000) J. Biol. Chem. 275, 21508–21513

12. Hwang, Y.-H., Tani, M., Nakagawa, T., Okino, N., and Ito, M. (2005) Biochem. Biophys. Res. Commun. 331, 37–42

13. Garcia-Sanchez, A., Cerrato, R., Larraza, J., Ambrose, N. C., Parra, A., Alonso, I. M., Hermoso-de-Mendoza, M., Rey, J. M., and Hermoso-de-Mendoza, J. (2004) Vet. Microbiol. 99, 67–74

14. Acharya, U., Patel, S., Koundakjian, E., Nagashima, K., Han, X., and Acharya, J. K. (2003) Science 299, 1740–1743

15. Acharya, U., Mowen, B. M., Nagashima, K., and Acharya, J. K. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 1922–1926

16. Bodey, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) Rev. Infect. Dis. 5, 279–313