Expression of a Functional Sphingomyelinase of *Pseudomonas* sp. TK4 in Mammalian Cells

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We report here the expression of a bacterial sphingomyelinase in mammalian cells as a functionally active form. A chimeric *Pseudomonas* sphingomyelinase fused with the lysosomal sorting motif of lysosomal acid phosphatase was sorted to lysosomes in mammalian cells. As expected, the chimeric SMase hydrolyzed sphingomyelin *in vivo* to produce ceramide, part of which was converted to glucosylceramide.

**Key words:** sphingomyelin; lysosomes; Niemann-Pick disease; sphingomyelinase; lysosomal acid phosphatase

Sphingomyelin (SM), consisting of a phosphorylcholine and a ceramide, is a ubiquitous component of the plasma membrane of vertebrates.1) Recently, SM on ectoplasmic membranes was found to be enriched with other sphingolipids and cholesterol to form microdomains that regulates various cellular events, such as signaling and membrane trafficking.2) In mammals, SM is eventually transported to lysosomes and catabolized by lysosomal acid sphingomyelinase (SMase). A genetic deficiency in acid SMase results in abnormal storage of SM in lysosomes, leading to Niemann-Pick disease.3) Trials aiming to decrease the accumulation of SM in lysosomes have been carried out over the past decade. Currently, the most effective and successful treatment for sphingolipid storage disease is an enzyme replacement therapy that compensates for the SMase activity lacking in patients.4) Gene therapy is also potentially useful, but basic data have yet to be fully accumulated. In this study, we attempted to express a bacterial SMase in lysosomes of mammalian cells by transfecting a fusion construct in which a DNA encoding *Pseudomonas* SMase5) was fused with that encoding the lysosomal sorting motif of acid phosphatase (LAP). As expected, chimeric SMase was delivered to the lysosomes and the enzyme degraded endogenous SM.

LAP is a lysosomal membrane protein composed of four different structural domains alone viz., a signal sequence, a catalytic domain, a transmembrane domain, and a cytoplasmic tail having a tyrosine motif (Fig. 1).6) It has been reported that both a transmembrane domain and a cytoplasmic tail are required for LAP to be delivered to lysosomes.6) To determine whether these domains of LAP work correctly when fused with other proteins, a chimeric protein with green fluorescent protein (GFP) was constructed in which the catalytic domain of LAP was replaced with GFP (Fig. 1). Briefly, a Not I–Not I fragment containing amino acid residues from Val361 to the C-terminus of rat LAP (kindly provided by Dr. Yoshitaka Tanaka, Graduate School of Pharmaceutical Sciences, Kyushu University) was inserted into the Not I sites of pCMV/myc/ER/GFP (Invitrogen, Carlsbad, CA). This chimeric GFP was then expressed in human embryonic kidney-derived HEK293 cells grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS, and 60 μg/ml of kanamycin in a humidified incubator containing 5% CO2. DNA transfection was carried out using LipofectAMINE™ PLUS (Invitrogen) according to the instructions of the manufacturer. After 24 h, the cells were fixed with 3% paraformaldehyde in PBS and analyzed with a confocal laser-scanning microscope. It was found that the fluorescent signal of the chimeric GFP was distributed in the perinuclear Golgi-like compartments, but was not co-localized with a lysosome marker, LysoTracker (Molecular Probes) (Fig. 2). However, the chimeric GFP was co-localized with LysoTracker when the transfected cells were grown in medium containing 1 mM leupeptin, a cystein protease inhibitor. This result suggests that the chimeric GFP was degraded by lysosomal proteases in the absence of the inhibitor though the protein was sorted to lysosomes. Hence it was concluded that fusion of the target protein with the transmembrane domain and the cytoplasmic tail of LAP causes the chimeric GFP-protein to express in the lysosomes in the presence of a protease inhibitor.
Next, a chimeric SMase construct in which the DNA encoding the enzyme (Tyr^{18} to C-terminus) was fused with that encoding GFP and that encoding the transmembrane domain and cytoplasmic tail (Tail) of LAP was generated (Fig. 1) and expressed in HEK293 cells in the presence of leupeptin. As shown in Fig. 2, the fluorescent signals of the chimeric SMase were merged with those for LysoTracker, and were also observed in Golgi-like compartments in the presence of leupeptin.

Chinese hamster ovary-derived (CHOP) cells transformed with a chimeric SMase construct were collected, and SMase activity in cell lysate and culture supernatant was measured using C6-NBD-SM as a substrate. The assay of SMase was performed as described previously. Briefly, the reaction mixture contained 500 pmol of C6-NBD-SM and cell lysate in 25 mM Tris–HCl buffer, pH 7.5, containing 0.1% Triton X-100 (w/v) and 5 mM MnCl$_2$. Following incubation at 37 °C for 30 min, reaction products were separated by TLC using chloroform/methanol/0.02%CaCl$_2$ (5/4/1, v/v/v) as a developing solvent, and quantified using a Shimadzu CS-9300 chromatoscan (excitation 475 nm, emission 525 nm). Activity was detected in the cell lysate but not in the medium, indicating that the chimeric enzyme was not detached from the cells under the conditions used (Fig. 3A). It is noteworthy that no detectable SMase activity was found in the cell lysate of mock transfectants when measured at pH 7.5 using C6-NBD-SM as the substrate.

Finally, to address whether endogenous SM can be
degraded by the enzyme, transformed cells with chimeric SMase gene were labeled with \[^{14}C\]palmitic acid (1 \(\mu Ci/ml\)), a precursor for the synthesis of SM. After cultivation at 37°C for 24 h, total lipids were extracted from the cells with chloroform/methanol (2/1, v/v), separated by TLC using chloroform/methanol/water (60/35/8, v/v/v) as a developing solvent, and analyzed with FLA-5000 and Imaging Gauge V3.0 (Fujifilm, Tokyo, Japan). Compared to mock transfectants, the intracellular content of \[^{14}C\]SM was found to be reduced by 60% in SMase transfectants (Fig. 3B, C) simultaneously with the generation of \[^{14}C\]ceramide (Fig. 3B), although it is currently unclear that lysosomal SM was actually hydrolyzed by the chimeric SMase. \[^{14}C\]Glucosylceramide also increased in SMase transfectants (Fig. 3B), possibly due to the conversion of SM-derived ceramide to glucosylceramide by the action of UDP-glucose:ceramide glucosyltransferase.7)

Type A and B Niemann-Pick diseases are lysosomal storage disorders caused by a defect in lysosomal acid SMase. Recently, enzyme replacement therapy was found to improve the symptoms of the disease. Here we report an alternative therapeutic strategy for the degradation of intracellular SM using a bacterial SMase gene. This study provides some insight into gene therapy for the sphingolipid storage diseases at the cellular level. However, whether a chimeric SMase can efficiently reduce the SM content in cells of patients with Niemann-Pick diseases remains to be elucidated. Furthermore, the results obtained here suggest that microbial genes encoding sphingolipid-degrading enzymes can be useful as a tool to elucidate the functions of sphingolipids and their metabolites in a specific organ-elle.

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