Acid Sphingomyelinase: Relation of ⁹³Lysine Residue on the Ratio of Intracellular to Secreted Enzyme Activity

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Acid sphingomyelinase (ASM) is the lysosomal enzyme responsible for the hydrolysis of sphingomyelin to ceramide and phosphocholine. An inherited deficiency of this enzymatic activity results in the Type A and B forms of Niemann-Pick disease (NPD). ASM is also readily secreted from cultured cells and can rapidly move from lysosomes to the cell surface upon stimulation by cytokines and other factors. Recent interest has focused on the role of this secreted/cell surface enzyme in ceramide-mediated signal transduction. We therefore sought to understand the mechanism(s) that might regulate intracellular targeting and secretion of this important hydrolase. Most lysosomal proteins are targeted to lysosomes in mammalian cells via the mannose 6-phosphate recognition system. Using cultured skin fibroblasts from I-cell disease patients, in which one of the enzymes responsible for mannose phosphorylation, GlcNAc-phosphotransferase, is deficient, we determined ASM activities in cell homogenates and media. The ratio of secreted to intracellular activity was ~8-fold greater in I-cell than in normal cells, indicating that mannose phosphorylation is important in the trafficking of this hydrolase. Most of the secreted activity required Zn⁺² for full activity, supporting the concept that intracellular exposure of ASM to zinc within lysosomes is required for enzymatic activation. The recognition of lysosomal proteins by GlcNAc-phosphotransferase is mediated by protein structure, and a specific three-dimensional arrangement of lysine residues exposed on the surface of several enzymes has been shown to be critical for mannose phosphorylation. Alanine scanning mutagenesis of thirteen lysine residues in ASM demonstrated that ⁹³lysine residue plays a critical role in ASM targeting since the K93A mutant had reduced intracellular activity, but enhanced secreted activity that was zinc responsive. ——— acid sphingomyelinase; I-cell disease; zinc dependent sphingomyelinase; alanine scanning mutagenesis; lysine residue

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Acid sphingomyelinase (sphingomyelin phosphodiesterase, EC 3.1.4.12, ASM) is a lysosomal enzyme responsible for the hydrolysis of sphingomyelin to ceramide and phosphocholine (Schuchman and Desnick 2001). ASM is important for sphingolipid turnover in cells, and ASM-mediated ceramide generation has emerged as a potentially important signaling pathway (Gulbins and Grassme 2002; Gulbins 2003; Goggel et al. 2004). Among several different forms of sphingomyelinase identified from mammalian sources, the acidic, Zn^{2+} -dependent ASM (Zn-ASM) was first shown to exist in fetal bovine serum by Spence et al. (1989). It was subsequently demonstrated that Zn-ASM is encoded by the same gene as lysosomal ASM, and that the Zn-ASM is secreted by many cell types, including macrophages, skin fibroblasts, and vascular endothelial cells (Schissel et al. 1996; Marathe et al. 1998). Furthermore, when endothelial cells were incubated with inflammatory cytokines, such as interleukin- 1β and interferon- γ , Zn-ASM secretion was increased ~2-3 fold above the already high level of basal secretion, whereas intracellular ASM activity decreased (Marathe et al. 1998). Thus, regulatory pathways are likely to exist that modulate the trafficking of ASM from lysosomes to the cell surface.

Most lysosomal proteins are targeted to lysosomes in mammalian cells via the mannose 6-phosphate recognition system (Kornfeld and Sly 2001). Phosphorylation of mannose residues on N-linked oligosaccharide chains of lysosomal proteins is carried out by two enzymes: UDP-N-acetylglucosamine: lysosomal enzyme N-acetylglucosaminyl-1-phosphotransferase and N-acetylglucosamine 1-phosphodiester-N-acetylglucosaminidase. The recognition of lysosomal proteins by these enzymes is mediated by their protein structure (Cuozzo et al. 1994, 1995, 1998; Tikkanen et al. 1997), and site directed mutagenesis/expression studies have revealed that individual lysine residues are critical for the recognition of three lysosomal enzymes by GlcNAc-phosphotransferase: cathepsin D, cathepsin L, and aspartylglucosaminidase (AGA). These results have suggested that a specific three-

dimensional arrangement of lysine residues exposed on the surface of lysosomal proteins is generally important for mannose phosphorylation.

To investigate the role of mannose phosphorylation on the intracellular processing of ASM, we first measured the levels of lysosomal and secreted ASM activity in the cultured fibroblasts from an I-cell disease patient, a disorder in which GlcNAc-phosphotransferase is deficient and newly synthesized lysosomal enzymes are secreted into the extracellular medium instead of being correctly targeted to the lysosomes. Second, we performed alanine scanning mutagenesis of all lysine residues of ASM to attempt to determine the recognition site for GlcNAc-phosphotransferase. In this report we identify a specific lysine residue that is important for ASM targeting and discuss several implications of these findings.

MATERIALS AND METHODS

Cells and growth conditions

COS-1 cells were purchased from the Riken Cell Bank and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Dainippon Pharmaceutical Co., Ltd., Tokyo), 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Normal human skin fibroblasts were obtained from three control individuals and I-cell disease fibroblasts were obtained from a male patient who was previously described (Kurobane et al. 1986). The fibroblasts were maintained in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and 10% fetal bovine serum. All cells were routinely grown in 75 cm^2 flasks (Corning Japan, Tokyo) in 5% CO_2 , and were used at full confluency at 37°C. To determine ASM activity in normal and I-cell disease fibroblasts, approximately 2×10^6 cells were plated in 10-cm dishes in DMEM containing FBS (10%), penicillin (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$) and L-glutamine (2 mM) and grown for 48 hr. The media was then changed to serum free media containing bovine serum albumin (0.2%), and the cells were grown for an additional 12 hr, washed 3 times with phosphate buffered saline (PBS), and finally incubated for 48 hr in fresh serum free medium containing bovine serum albumin (0.2%). This 48 hr "conditioned medium" was collected to determine ASM activity.

Mutagenesis studies

To introduce mutations into each of the lysine codons of the full-length ASM cDNA, the PCR overlap method was used (Takahashi et al. 1992). Primers were designed to substitute alanine for lysine residues in the protein. The AAA codons of Lys-93, Lys-187, and Lys-418 were changed to GCA, and the AAG codons of Lys-105, Lys-106, Lys-118, Lys-124, Lys-183, Lys-249, Lys-305, Lys-347, Lys-433, Lys-576 were changed to GCG (Fig. 1). The mutations were inserted into a pGEM-7Zf(+) plasmid (Promega, Madison, WI, USA) that contained the full-length ASM cDNA. Mutagenized DNAs were sequenced in both orientations to confirm the presence of the mutations.

Expression of the wild-type and mutated ASM cDNAs

For transient expression in COS-1 cells, the mutated cDNAs were subcloned into the eukaryotic expression vector, p91023(B). About 2×10^6 COS-1 cells were plated on 10 cm dishes the day before transfection. Twelve μg of each mutated construct or wild-type construct were then transfected into COS-1 cells using 24 μl of the TransIT-LT1 reagent (Mirus Corporation, Madison, WI, USA). Negative controls for the transfections contained either no DNA or vector alone. The transfections were performed in serum free DMEM medium. The transfected cells were harvested after 48 hr and the ASM activities were determined.

SDS-polyacrilamide gel electrophoresis and immunoblotting

Protein samples, 30 μg each, were boiled in buffer containing 2% SDS and 5% β -mercaptoethanol for 10 min, loaded onto 10% gradient polyacrilamide gels, and electrophoresed for 90 min at 20 mA in buffer containing 0.1% SDS (SDS-PAGE). After SDS-PAGE, the proteins

were electrophoretically transferred (60 V for 4.0 h) onto nitrocellulose for immunoblotting. For immunoblotting, the nitrocellulose membranes were incubated with 0.2% non-fat dry milk in TBS buffer (20 mM Tris, 500 mM NaCl, pH 7.5) for 1 h at room temperature. The membranes were then incubated with rabbit anti-human acid sphingomyelinase polyclonal antiserum (1:200) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in antibody buffer (0.2% non-fat dry milk, 20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5). After washing twice with TTBS buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5), the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG for 1 h in antibody buffer at room temperature. The membranes were subsequently washed twice with TTBS buffer. Finally, the blots were soaked in the enhanced chemiluminescence reagent (Bio-Rad Laboratories, Tokyo) for 2 min and exposed to x-ray film for 1 min.

ASM enzyme assays

Forty-eight hrs following transfection, the conditioned media was collected and the cells were washed two times with PBS and harvested with a rubber policeman. The scraped cells were disrupted by sonication on ice using three 15-second bursts, and cellular homogenates were assayed for total protein by the method of Lowry et al. (1951) and for ASM activity as described below. The conditioned media were spun at 800 x g for 5 min to pellet any contaminating cells.

Assays for the ASM activity were carried out as follows: ^{14}C -sphingomyelin was purchased from NEN Life Science Products (Boston, MA, USA). The standard 200 μl assay mixture consisted of 100 μl of sample and 50 μl of assay buffer containing 4% Triton X-100 (1.0 M sodium acetate, pH 5.0; final concentration of Triton X-100 in the 200 μl assay mix, 1%). The reaction was initiated

Fig. 1. The position of lysine residues in ASM.

by the addition of 50 μ l of substrate (20 nmol, 14 C-sphingomyelin, 0.08 μ Ci/20 nmol) in 0.2% taurodeoxycholic acid. When added, the final concentrations of EDTA and Zn^{2+} were 0.02 mM and 0.1 mM, respectively. The assay mixtures were incubated at 37°C for 1 hr and quantified by the method of Chatterjee and Ghosh (1991).

RESULTS

ASM activity in I-cell disease skin fibroblasts

Intracellular and secreted ASM activities were determined in cultured skin fibroblasts from an I-cell disease patient. The levels of intracellular ASM activity decreased about 2-fold in the I-cell disease fibroblasts compared with normal fibroblasts (28.1 ± 2.0 nmol/mg/hr and 54.0 ± 8.8 nmol/mg/hr, respectively) (Fig. 2A). In contrast, the I-cell disease fibroblasts had markedly elevated zinc-responsive ASM activity in the medium (233.8 ± 56.9 nmol/mg/hr and 59.6 ± 2.7 nmol/mg/hr, respectively) (Fig. 2B). Thus, the ratio of secreted to intracellular ASM activities was about 8-fold greater in I-cell vs normal fibroblasts.

Mutagenesis/expression studies

Thirteen lysine residues in ASM were individually converted to alanine residues by site-directed mutagenesis and the resulting mutated cDNAs were transfected into COS-1 cells for transient expression. Intracellular and secreted ASM activities of wild type were about 16 times and 8 times of the controls (both no DNA and vector alone), respectively. The results of ASM activity are shown as percentage relative to the wild type ratio (Fig. 3). From these studies five groups of mutants were identified (1) those that did not result in any altered ASM activity (K104A), (2) those in which the intracellular and secreted ASM activities were both reduced (K249A, K305A, K419A, K433A, K576A), (3) those in which the intracellular activity was not reduced, but the secreted activity was reduced (K105A, K118A, K124A, K183A), (4) those in which the intracellular activity was reduced, but the secreted activity was unchanged (K187A, K347A) and (5) those in which the intracellular activity was reduced, but the secreted activity was increased (K93A).

Fig. 2. Intracellular ASM and secreted ASM activities of fibroblasts with I-cell disease. A: ASM activity in cell homogenate. Two types of assay buffer, containing EDTA or Zn^{2+} , were used for the measurement. B: ASM activity in conditioned medium. The medium of I-cell disease demonstrated higher ASM activity than that of normal fibroblasts. Data are expressed as the mean $\pm$ S.D. * $p < 0.01$ ($n = 3$).

The fact that the K93A mutant had reduced intracellular activity, but increased zinc responsive, secreted activity suggested that this mutation had altered normal ASM trafficking without affecting overall activity. To confirm this observation three independent transfection experiments were carried out using this mutant. Western analysis of ASM protein expressed from the wild-type and K93A ASM cDNAs in COS-1 cells showed one significant band on both lanes of molecular

A. Cell homogenate

B. Conditioned medium

Fig. 3. ASM activities of the COS-1 cells and conditioned media transfected with mutated ASM cDNAs. A: Cell homogenate. B: Conditioned medium. The ASM activities were expressed as percentage relative to the wild type ratio.

size 52-kDa (Fig. 4). This band corresponded to the lysosomal ASM protein reported by Marathe et al. (1998).

As shown in Figs. 5A and 5B, the K93A mutant had ~60% of normal intracellular ASM activity (182.7 ± 31.2 nmol/mg/hr and 303.0 ± 22.1 nmol/mg/hr, respectively) and ~120% of normal zinc responsive, secreted ASM activity (478.1 ± 112.6 nmol/mg/hr and 407.3 ± 72.8 nmol/mg/hr, respectively). Thus, the ratio of secreted to intracellular ASM activity in the K93A mutant was ~2-fold higher when compared to wild-type.

DISCUSSION

Skin fibroblasts from an I-cell disease patient had reduced intracellular ASM activity and a corresponding increase in secreted activity, similar to what had been found for other lysosomal enzymes that utilize the mannose 6-phosphate targeting system (Kornfeld and Sly 2001). This result not only demonstrates that the mannose 6-phosphate system was used by this hydrolase, but also supports the notion that under normal circumstances, ASM is only active once it reaches lysosomes and comes in contact with zinc cations (since the in-

Fig. 4. Immunoblot analysis of ASM expressed in COS-1 cells. Western analysis of ASM protein expressed from the wild-type (lane 1) and K93A (lane 2) ASM cDNAs in COS-1 cells showed one significant band on both lanes at a molecular size of 52-kDa. Lane 3 shows immunoblot analysis of the COS-1 cells without expression study. Protein samples, 30 μ g each, were loaded for SDS-PAGE.

crease in secreted activity could only be detected in I-cell fibroblasts once zinc cations were added to the assay mixture). The ASM gene gives rise to a common mannosylated precursor protein, which is shuttled into either the lysosomal trafficking pathway or the secretory pathway (Schissel et al. 1996). Since it contains several highly conserved zinc-binding motifs, it has been suggested that when the enzyme is exposed to cellular pools of Zn^{2+} , most likely within the lysosomes, it becomes activated. However, this exposure/activation does not occur in the secretory pathway, thus explaining the requirement for exogenous Zn^{2+} for the detection of secreted ASM activity. Our results are consistent with this hypothesis since in I-cell fibroblasts some of the ASM molecules will not be properly targeted to lysosomes and thus enter the secretory pathway by default. These molecules should not be active in the media until they are exposed to exogenous zinc, which is what we observed. Our results are

A. ASM activity in cell homogenate (Zn^{2+} buffer)

B. ASM activity in conditioned medium

Fig. 5. Intracellular ASM and secreted ASM activities of COS-1 cells transfected with ASM cDNAs. A: ASM activity in cell homogenate (Zn^{2+} buffer). ASM activities of cell homogenates from COS-1 cells transfected with wild-type or K93 mutant ASM cDNA was determined using Zn^{2+} buffer. B: ASM activity in conditioned medium. ASM activities of the medium from COS-1 cells transfected with wild-type or K93 mutant ASM cDNA were determined using Zn^{2+} and EDTA buffer. Data are expressed as the mean $\pm$ S.D. * $p < 0.01$ ($n = 3$), ** $p < 0.05$ ($n = 3$).

partly supported by previous studies, in which the Zn^{2+} -dependency of ASM was not discussed (Wenger et al. 1976; Hurwits et al. 1994).

Expression studies of thirteen mutated ASM cDNAs also demonstrated that the substitution of alanine for lysine at position 93 decreased intracellular ASM activity and increased secreted activity. Although there was a difference in respon-

siveness of the secreted ASM to zinc between the I-cell patient cells and the COS-1 cells transiently transfected with K93A ASM cDNA, it may be ascribed to the transient expression system. In fact, COS-1 cells transiently transfected with wild-type ASM cDNA secreted most of the zinc-non-responsive ASM enzymes in the media. Another expression system, such as stable expression system, may give us further elucidation of the ASM zinc dependency in the medium. Again, the increased secreted activity was zinc responsive in the COS-1 cells transfected with K93A ASM cDNA. We propose that this is due to altered mannose 6-phosphate targeting and suggest that ⁹³lysine plays an important role in the recognition of human ASM by GlcNac-phosphotransferase. In our experiment, we did not directly evaluate the phosphorylation of K93A ASM by GlcNac-phosphotransferase. Decrease of the phosphorylation should be ascertained in K93A ASM protein by another experiment.

The recognition sites of GlcNac-phosphotransferase by three other lysosomal proteins, cathepsin L, cathepsin D, and aspartylglucosaminidase (AGA), have been previously examined in some detail (Cuozzo et al. 1994, 1995, 1998; Tikkanen et al. 1997). Those results indicated that the phosphotransferase recognition sites were composed of lysine residues brought together on the surface of the protein following folding and assumption of the proper three-dimensional structure. Mutagenesis of cathepsin D and cathepsin L indicated that a pair of lysine residues (⁵⁴lysine and ⁹⁹lysine in cathepsin L, and ²⁰³lysine and ²⁹³lysine in cathepsin D) play a major role in the phosphorylation of each respective enzyme (Cuozzo et al. 1995, 1998). Structural comparison of the two proteins also revealed some similarities. The lysine residues were spaced 34Å apart and were exposed on the surface of the protein and positioned in a specific orientation relative to the target oligosaccharide. Those characteristics may be a general model for the GlcNac-phosphotransferase recognition signals of other lysosomal proteins. Analysis of AGA also revealed that three lysine residues, ¹⁷⁷lysine, ¹⁸³lysine, and ²¹⁴lysine, were important for proper phosphorylation

of the oligosaccharides (Tikkanen et al. 1997). On the basis of the three-dimensional structure of AGA, these three lysines clearly protrude from the surface of AGA. Since the crystal structure of ASM has not yet been determined, we are not able at this time to demonstrate whether ⁹³lysine residues are on the surface or their physical relationship to the five oligosaccharide chains.

The function of secreted ASM has been the subject of recent investigations, but specific functions have not yet been elucidated. However, a wide variety of cell types have been shown to secrete ASM activity to varying degrees (Schissel et al. 1996). For example, J774 macrophages and mouse peritoneal macrophages have higher secretory rates (over 30%) than human skin fibroblasts and monocytes (about 10%). Another report showed that cultured human endothelial cells obtained from the coronary artery, as well as umbilical vein endothelial cells, secreted massive amounts of ASM (up to 20-fold more than macrophages) (Marathe et al. 1998). This latter result is notable since ASM-generated ceramide has been implicated in endothelial apoptosis, and it could be that secreted ASM is responsible for this pathway. If secreted ASM activity is important in this and other cellular events it would therefore seem logical to suggest that cells should have mechanisms by which to regulate the levels of secreted and intracellular enzymes. Indeed, the secretion rates can be influenced by regulatory factors, such as cytokines (Marathe et al. 1998), and different cell types have different basal secretion rates, supporting the notion that regulation of ASM targeting is both physiological and important. Lysine-93 may be important in this regard by modulating the enzyme's recognition/modification by GlcNac-phosphotransferase. This also suggests that it may be an important target for pharmacologic intervention when upregulation of secreted ASM activity is desired.

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