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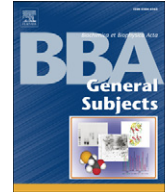

Critical determinants of mitochondria-associated neutral sphingomyelinase (MA-nSMase) for mitochondrial localization

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Background: A novel murine mitochondria-associated neutral sphingomyelinase (MA-nSMase) has been recently cloned and partially characterized. The subcellular localization of the enzyme was found to be predominant in mitochondria. In this work, the determinants of mitochondrial localization and its topology were investigated.
Methods: MA-nSMase mutants lacking consecutive regions and fusion proteins of GFP with truncated MA-nSMase regions were constructed and expressed in MCF-7 cells. Its localization was analyzed using confocal microscopy and sub-cellular fractionation methods. The sub-mitochondrial localization of MA-nSMase was determined using protease protection assay on isolated mitochondria.
Results: The results initially showed that a putative mitochondrial localization signal (MLS), homologous to an MLS in the zebra-fish mitochondrial SMase is not necessary for the mitochondrial localization of the murine MA-nSMase. Evidence is provided to the presence of two regions in MA-nSMase that are sufficient for mitochondrial localization: a signal sequence (amino acids 24–56) that is responsible for the mitochondrial localization and an additional 'signal-anchor' sequence (amino acids 77–99) that anchors the protein to the mitochondrial membrane. This protein is topologically located in the outer mitochondrial membrane where both the C and N-termini remain exposed to the cytosol.
Conclusions: MA-nSMase is a membrane anchored protein with a MLS and a signal-anchor sequence at its N-terminal to localize it to the outer mitochondrial membrane.
General significance: Mitochondrial sphingolipids have been reported to play a critical role in cellular viability. This study opens a new window to investigate their cellular functions, and to define novel therapeutic targets.
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1. Introduction

The mitochondrion is emerging as a novel compartment of ceramide metabolism and function. Mitochondria have been shown to contain many sphingolipids including sphingomyelin (SM) and ceramide [1,2]. Many ceramide generating enzymes have been suggested to reside in this organelle, including ceramide synthases (CerS1, CerS2, CerS4 and CerS6) [3–5], zebrafish and mouse neutral sphingomyelinases [6,7], and neutral ceramidases [8].

Abbreviations: SMase, sphingomyelinase; N-SMase, neutral sphingomyelinase; SMPD, sphingomyelin phosphodiesterase; SM, sphingomyelin; MAMS, mitochondria-associated membranes; CerS, ceramide synthase; Hsp, heat shock protein; MLS, mitochondrial localization signal; TMD, transmembrane domain; ER, endoplasmic reticulum; GFP, green fluorescent protein; bSMase, bacterial sphingomyelinase

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Besides the occurrence of these enzymes, various studies have also suggested the biological significance of ceramide generation in this compartment. Birbes et al. [9] showed that the selective targeting of bacterial sphingomyelinase to mitochondria and not to other compartments resulted in apoptosis, and over-expression of Bcl-2 prevented these effects [9]. Dai et al. showed that UV-induced apoptosis is marked by an increase in SM in all sub-cellular locations particularly in mitochondria in HeLa cells, and ceramide level was found to be elevated in mitochondria at 2–6 h, consistent with cell death time course. D609, an inhibitor of sphingomyelin synthase, rescued the cells from the spike in SM and ceramide and consequently cell death [10] suggesting the involvement of the SM hydrolysis in the cell death triggered by UV irradiation.

In another study in *Caenorhabditis elegans*, subsequent to inactivation of ceramide synthase, somatic apoptosis was unaffected but ionizing radiation-induced apoptosis of germ cells was obliterated, and this phenotype was reversed by microinjection of long-chain natural ceramide. Radiation induced ceramide accumulation in mitochondria and consequent activation of CED-3 caspase and apoptosis [11].

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In the studies on isolated mitochondria, exogenous synthetic N-acetylsphingosine (C2-ceramide) elicited inhibition of state 4 respiration (respiration upon exhaustion of ADP) and inhibition of electron transport complex I [12]. In another study, respiratory chain complex III function was reduced by C2-ceramide whereas N-acetylsphinganine (C2-dihydroceramide), which lacks the functional double bond, did not alter mitochondrial respiration or complex III activity [13].

Sphingomyelinases (SMases) catalyze the hydrolysis of SM to ceramide. There are three major classes of SMases depending on their optimum pH of activity, acid, neutral, and alkaline SMases. Acid SMase (SMPD1) exists in both the lysosomal and secretory forms; both of them operate at a pH optima of ~5; deficiency of this enzyme causes Niemann–Pick disease. Alkaline SMase is found in the mucosa of the gastrointestinal tract and bile in humans; it has a pH optima of 9; it plays a role in dietary SM digestion but also displays broad substrate specificity [14].

Neutral sphingomyelinases operate at pH optima of ~7. Mouse and human nSMase 1 (SMPD2), first cloned in 1998, [15] were found to be ubiquitously expressed in various tissues and co-localized with ER and Golgi [16]. Human nSMase 2 (SMPD 3) was found to be localized in the Golgi and plasma membrane [17]. Human nSMase 3 (SMPD4), although not a member of the neutral SMase family due to the lack of sequence similarity, it biochemically ‘behaved’ like neutral sphingomyelinase; it was found to be a C-tail anchored transmembrane protein [18], and enriched in the ER [19]. The family also includes Isc1p from *S. cerevisiae* [20] and its homologue css1 in *Schizosaccharomyces pombe* [21].

Among the sphingomyelinases identified so far, the zebrafish mitochondrial SMase was the first SMase found in the mitochondria to be cloned and characterized. Over-expression of this protein in HEK293 cell lines localized it to mitochondria whereas mutants lacking the first N-terminal 35 residues did not localize to mitochondria. Topologically, it was found to be in the mitochondrial inter membrane space and/or inner membrane of zebrafish embryonic cells [6]. Murine mitochondria associated sphingomyelinase (MA-nSMase) was the second mitochondrial member of this family [7]. In our previous work, it was found that MA-nSMase retained a significant number of conserved amino acids that are necessary for cation binding; a P-loop like domain, and two critical residues, D470 and H471 required for its catalytic activity. The sequence homology with zebrafish reveals a putative mitochondrial localization signal (MLS) extending from 24 to 56aa, and a putative transmembrane domain (TMD) extending from 77 to 99aa. Biochemical characterization of MA-nSMase using lysates from transiently transfected HEK293 cells disclosed that the murine MA-nSMase belongs to the neutral class of sphingomyelinases [7].

The majority of mitochondrial proteins are synthesized in the cytosol and imported into mitochondria. These precursor proteins are thought to be stabilized by chaperones, especially Hsp70 and Hsp90 [22,23]. Normally, these precursors contain a presequence, a signal at the most N-terminal region of the protein (or matrix-targeting sequences or MTS) to target the protein to mitochondria. In most cases, the presequence is cleaved after reaching the matrix by mitochondrial-processing peptidase (MPP) [24,25]. However, there are many mitochondrial proteins that do not have an N-terminal signal but rather possess an internal targeting signal. Precursors of mitochondrial outer membrane proteins have such a signal. Outer membrane proteins might be N-terminally anchored as in Tom 20 and Tom 70 or C-terminally anchored as in Tom5 and Bcl2 [26]. A third type spans the outer membrane twice with a small loop in the intermembrane space as in Fzo 1 [27]. Porins and Tom 40 traverse the outer-membrane many times forming β barrel structure [28]. N-terminal anchored proteins are called ‘signal anchored’ proteins because the transmembrane domain TMD and the residues flanking it serve for both its intracellular sorting and anchoring functions [29]. Signal anchored proteins do not share sequence similarity but they may be characterized by certain features such as moderate hydrophobicity of its transmembrane domain and positively charged C terminal residues flanking the TMD as in Tom20 [30]. Also, at least three basic amino acids should be flanking the TMD for membrane anchorage as in Tom70 [31]. The salient features of C-terminally anchored proteins are relatively short TMD with moderate hydrophobicity and positive charged residues at its flanking regions. The relative contribution of each of these structural features seems to vary from protein to protein. For instance, a net positive charge flanking the TMD is extremely important for Bcl-2 and Bcl-X_L. The TMD of both proteins has similar hydrophobicity and length. However, two positively charged amino acids at either side of TMD in Bcl-x_L make it go exclusively to mitochondria [32] whereas in Bcl2-one positive charge on either side of the TMD makes it indiscriminately targeted to mitochondria, ER and nuclear envelopes. The targeting signals of many of these outer membrane proteins have previously been reviewed [33].

Since MA-nSMase appeared to localize to the mitochondrion which is a critical organelle involved in the cell survival and growth, this study was undertaken to define the critical domain in the protein that targets it to the mitochondria and define its topology.

2. Materials and methods

2.1. Site directed mutagenesis and generation of fusion constructs

For making all deletion mutant constructs except Δ24–99, Quickchange site-directed mutagenesis kit (Stratagene, Catalogue # 200518) was employed as per the manufacturer's instruction. For generating Δ24-99 construct and bacterial SMase fusion constructs, the overlap extension method was followed as described previously [34]. This was a two-step cloning technique. It was based on performing two independent PCR reactions with partially matching overhangs. In the subsequent fusion reaction, the PCR products of the previous two reactions were used as templates in which the overlapping ends anneal allowing the 3′ end of each strand acting as primer for 3′ extension of the complementary strand. The most upstream forward primer and the most downstream reverse primer (primers are listed in Supplemental section Table 2) serve as primers to amplify the full length fused product. Taq polymerase was used to perform the third PCR to leave an ‘A’ overhang which was gel purified and subcloned into pEF-6/V5-His TOPO TA expression kit from Invitrogen (Catalogue #K9610-20). For making the GFP constructs, forward and reverse primers (primers are listed in Supplemental section Table 3) with restriction sites (Kpn I/HindIII respectively) were used to generate the desired products and subcloned into pEGFP-N1 plasmid from Clontech (Catalogue #6085-1).

For bacterial SMase (bSMase) fusion constructs, the catalytic domain of bSMase was used. Bioinformatics prediction using the SignalP 4.1 software suggested that there is a signal peptide within the first 1–27 residues of the protein followed by a putative cleavage site between residues 27 and 28. Thus, to make the fusion constructs and to prevent protein mistargeting, we deleted the first 27 amino acids of the bacterial SMase protein.

For generation of fusion proteins with tags at different locations, the overlap extension method as described previously was used with primers carrying sequences for the V5 tags (primers are listed in Supplemental section Table 4) and restriction enzyme sites corresponding to Bam H1/Pme I. The resultant PCR products were subjected to restriction enzyme digestion, gel purified, and then subcloned into pEF-6/V5-His vector. For generating the truncation fusion constructs 1–27, 1–66 and 1–198, PCR was done to amplify the desired length product and the resultant construct was subcloned into pEF-6/V5-His TOPO TA expression kit as mentioned previously. For making of 1–45 and 1–56, PCR was done using 1–66 fusion construct as a template with primers having restriction site for BamH I/ Not I (primers are listed in Supplemental section Table 1). The resultant PCR products were subjected to restriction enzyme digestion, gel purification, and subcloning into pEF-6/V5-His vector. The authenticity of these constructs was verified

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