Occurrence of two molecular forms of human acid sphingomyelinase

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Human acid sphingomyelinase (ASM) hydrolyses sphingomyelin to ceramide and phosphocholine. Metabolic studies on COS-1 cells transfected with ASM cDNA revealed the occurrence of an enzymically inactive precursor which is differentially processed to two predominant native glycoprotein forms: a 70 kDa polypeptide corresponding to human urinary protein and a 57 kDa form. Formation of these potentially active forms was shown to be restricted to distinct compartments. Maturation of the ASM precursor to a predominant 70 kDa form occurs exclusively inside acidic organelles, whereas variable amounts of 57 kDa ASM are detectable immediately after biosynthesis. Metabolic labelling of transfected COS-1 cells with [^{32}P]P_i further suggests that this form obviously does not carry oligomannosyl-phosphate residues, in contrast with the mature lysosomal ASM. In order to verify that this early form of active ASM results from co-post-translational proteolysis of the ASM precursor and not from the use of different translation-initiation sites on the ASM mRNA, appropriate 5′-mutagenized cDNA constructs were transiently expressed. These results clearly indicate that the first potential in-frame AUG is exclusively used for translation initiation *in vivo* and that deletion of the proposed signal sequence for endoplasmic reticulum import completely eliminates the ability of the translation product to enter the vacuolar apparatus. As there are two different subcellular sites of maturation of the ASM precursor, and intracellular targeting of the two processed forms appears to be different, the two ASM proteins may contribute to distinct physiological functions.

INTRODUCTION

Human acid sphingomyelinase (ASM; EC 3.1.4.12) is a lysosomal glycoprotein which hydrolyses sphingomyelin to ceramide and phosphocholine. Since it became clear that an inborn error of sphingomyelin metabolism, known as Niemann–Pick disease, is caused by autosomal recessive deficiency of this enzyme (Brady et al., 1966), several groups have attempted to isolate it. Purification procedures starting from human placenta, spleen, brain and urine yielded inhomogeneous preparations of different sizes as well as widely diverging specific activities (Jones et al., 1981; Yamanaka and Suzuki, 1982; Sakuragawa, 1982; Weitz et al., 1985). This broad heterogeneity of apparently pure enzymes, ranging from 30 kDa up to 90 kDa, and the proposal of several potential subunits contributing to active enzyme complexes, has led to controversy over the past 10 years. Thus far, reports on enzyme-specific antibodies and provisional biosynthetic studies have not led to unequivocal experiments (Rousson et al., 1987; Jobb and Callahan, 1989). In 1987 we finally succeeded in purifying ASM to homogeneity from the urine of patients seriously affected by peritonitis, traumata or postcardiac surgery (Quintern et al., 1989a). This preparation yielded a monomeric glycoprotein about 70 kDa in size with a specific enzyme activity of 2.5 mmol/h per mg at pH 5.0. Amino acid sequencing led to isolation of three alternatively spliced cDNA species and information about the organization of the corresponding gene (Quintern et al., 1989b; Schuchman et al., 1991, 1992). The above data have been used as the basis for various mutation studies on mRNA and genomic DNA from cells of patients with Niemann–Pick disease (Levran et al., 1991a,b; Ferlinz et al., 1991; Takahashi et al., 1992; Vanier et al., 1993).

Renewed interest in sphingomyelin metabolism arose when several groups demonstrated that its degradation products potentially play a role in signal transduction. The discovery that regulation of cell differentiation and proliferation directly depends on the levels of ceramide, ceramide 1-phosphate and certain sphingoid bases in the cell triggered studies on sphingomyelin turnover. Further evidence of highly regulated sphingomyelin catabolism suggests a pivotal role for sphingomyelinase in the 'sphingomyelin cycle'. Degradation of sphingomyelin was found to occur in response to various cellular signals, such as tumour necrosis factor α, interleukin-1 and interferons (Mathias et al., 1991; Dobrowsky and Hannun, 1992). Moreover, recent findings indicate that an ASM besides the neutral sphingomyelinase, which has also been discussed as a potential ceramide-releasing enzyme, may be involved in this regulatory pathway which is proposed to be a cascade induced by tumour necrosis factor α acting by receptor-mediated stimulation of phosphatidylcholine-specific phospholipase C (Schütze et al., 1992). Released 1,2-diacylglycerol is postulated to activate sphingomyelinase under acidic conditions leading to elevated intracellular levels of ceramide which finally activate nuclear factor κB via ceramide-specific kinases. Intriguingly, pure human ASM, the only sphingomyelin-specific acidic lipase characterized so far, has previously been shown to be significantly stimulated by 1,2-diacylglycerol (Quintern et al., 1989a), thus pointing to a close relationship, if not identity, between ASM and the enzyme involved in this postulated sphingomyelin cycle. Therefore we raised appropriate anti-ASM antibodies in order to analyse further the biosynthesis and processing of this enzyme, its subcellular localization and targeting.

EXPERIMENTAL

Reagents

Restriction and modifying enzymes were purchased from

Abbreviations used: ASM, acid sphingomyelinase; COS-1, CV1 origin simian virus 40; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*NNN*-trimethylammonium methyl sulphate; DTAF, 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein; ER, endoplasmic reticulum; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; TBS, Tris-buffered saline; Endo F, β-endo-*N*-acetylglucosaminidase F.

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Table 1 Oligonucleotide sequences

The co-ordinates (base numbers) are numbered by counting from the adenine residue of the first initiation codon. Bold-letter bases refer to sequences altered to create restriction sites. Lower-case letters signify bases inserted to create a new initiation codon.

Oligonucleotide	Co-ordinates	Sequence
α1	7–25	5′-**CTGAATT**CGCTACGGAGCGTCACTCC-3′
β1sig	140–159	5′**GTGAATTC**aTGTCTGACTCTCGGGTTCTC-3′
γ1fs	−7–6	5′-**GTGAATT**CGCGACAATGTCCCCGCTACGGAG-3′
N2	647–629	5′-CCCTCCAGGTAGTCATGAT-3′

Boehringer-Mannheim and Bethesda Research Laboratories. DNA *Taq* polymerase was from Cetus. DNA-sequencing kits (Sequenase version 2.0) were obtained from United States Biochemicals. Plasmids for prokaryotic expression, *Escherichia coli* host strain M15 and Ni^{2+}/nitrilotriacetic acid–agarose were from Diagen. *N*-[1-(2,3-Dioleoyloxy)propyl]-*NNN*-trimethylammonium methyl sulphate (DOTAP) reagent for lipofection was purchased from Boehringer-Mannheim and 5-[(4,6-dichlorotriazin-2-yl)amino]fluorescein (DTAF)-labelled Fab fragments of goat anti-rabbit IgGs were from Dianova. Radiolabelled chemicals were obtained from Amersham. Culture media for bacteria and eukaryotic cells were from Gibco–BRL. All other commercially available materials were obtained from the following suppliers: Sigma, Merck, Pharmacia LKB Biotechnologies and New England Biolabs. Oligonucleotides were synthesized by MWG-Biotech.

Expression of ASM protein in *E. coli* and immunization of rabbits

The full-length cDNA of ASM except the portion containing the signal peptide was cloned into the prokaryotic expression vector pQE (Diagen). Expression of the corresponding cDNA construct yielded a fusion protein consisting of six N-terminal histidine residues followed by two additional amino acids derived from sequences of the cloning site and the ASM amino acid sequence representing the mature protein from residue 47 to 629. Purification of the fusion protein was easily achieved by nickel ion chelating chromatography (Hochuli et al., 1988). Polyclonal anti-ASM serum was raised in rabbits by injection of 100 μg of pure protein in combination with Freund's complete adjuvant followed by three injections of 50 μg of protein in Freund's incomplete adjuvant given at 4-week intervals. Animals were bled 10 days after the final injection.

Mutagenic ASM plasmids for transient expression in COS-1 cells

Eukaryotic expression vectors containing normal and mutant ASM cDNAs were constructed to characterize the properties and targeting of the resulting recombinant proteins. Full-length cDNA ligated with the *Eco*RI cloning site of p91023(B) (Kaufman, 1985) (designated wild-type ASM) was used to generate all mutagenized constructs discussed in this study (see Figure 5).

The α ASM construct missing the first ATG was created by PCR amplification using the primer α1 located downstream of the first ATG and containing an artificial *Eco*RI site as well as the upstream primer N2 (Table 1). The resultant PCR DNA was digested with *Eco*RI and *Cel*II and substituted for the corresponding wild-type area by cassette exchange.

The β ASM construct lacking both natural ATGs and the DNA region encoding the endoplasmic reticulum (ER) import signal was generated by PCR amplification of wild-type cDNA using a sense primer downstream of this sequence containing a 5′-flanking *Eco*RI site as well as an artificial ATG for translation initiation (β1sig) and the antisense primer N2. Construction of the mutant vector was also performed by transfer of a mutagenized *Eco*RI–*Cel*II cassette.

γ ASM containing a single base insertion between the first and second in-frame ATG was generated by PCR amplification of wild-type DNA using the frameshift oligonucleotide γ1fs and the antisense primer N2. The final vector γ ASM was constructed as described above. Standard procedures for molecular cloning were performed as described by Sambrook et al. (1989). All PCR-mutagenized sequences as well as DNA portions flanking the cloning sites were verified by the Sanger dideoxy-chain-termination method (Sanger et al., 1977).

Cell culture, transfection of COS-1 cells and ASM assay

About 5×10^5 COS-1 cells supplemented with Dulbecco's modified Eagle's medium (DMEM) and 10% fetal calf serum (FCS) were plated on 6 cm dishes the day before transfection. The standard liposome-mediated transfections with DOTAP were carried out using 7 μg of pure DNA for each dish. During this 6 h period the cells were maintained in OptiMEM (Gibco–BRL) medium without supplements. At 48 h after transfection, the cells were harvested and assayed for ASM activity using [^{3}H]choline-labelled sphingomyelin as described elsewhere (Quintern et al., 1987) or were plated on to coverslips for fluorescence microscopy.

Metabolic labelling and immunoprecipitation

About 48 h after transfection, COS-1 cells (21 cm^2 culture dishes) were starved for 1 h in 1 ml of methionine-free MEM containing 4% dialysed and heat-inactivated FCS. After 1 h, 100 μCi of L-[^{35}S]methionine (Amersham) was added and cells were labelled for the times indicated. The chase periods were started by the addition of unlabelled methionine (final concentration 100 μM). Experiments using brefeldin A were performed by adding the drug to a final concentration of 1 μg/ml to the culture medium 1 h before methionine starvation. Incubation with brefeldin A was continued throughout pulse and chase periods. For labelling with [^{32}]P_i, cells were incubated in DMEM minus phosphate with 4% dialysed FCS and 200 μCi of sodium [^{32}P]phosphate (> 9000 Ci/mmol) for 4 h. Labelling with 150 μCi of D-[2,6-^{3}H]mannose (30–60 Ci/mmol) was performed in DMEM containing 1 mM glucose and 4% dialysed FCS for 4 h.

The labelled COS-1 cells were rinsed once with Tris-buffered saline (TBS), harvested by scraping off the culture dishes and lysed in 0.5 ml of TBS buffer containing 1% Nonidet P40, 10 mM EDTA, 2 mM phenylmethanesulphonyl fluoride, 1 mM pepstatin and 1 mM leupeptin. For phosphate labelling, pyrophosphate, NaF and orthovanadate at final concentrations of 10, 5 and 1 mM respectively were added.

Cell extracts and media were preabsorbed with 10 μl of preimmune rabbit serum and 30 μl of a 50% (w/v) suspension of Protein A–Sepharose for 3 h. Immunocomplexes were removed by centrifugation at 300 ***g*** for 5 min.

The preabsorbed homogenates and media were centrifuged at 100000 ***g*** for 1 h in a Beckman Ti75 rotor. The supernatants were carefully removed and 2 μl of anti-ASM serum was added. They were then incubated for 12 h at 4 °C. Immunoprecipitation was carried out in the presence of 15 μl of Protein A–Sepharose

(50% suspension) for 2 h. Immunocomplexes were treated as described (Schnabel et al., 1992). Deglycosylation with EndoF (New England Biolabs) was performed according to the manufacturer's instructions.

Isoelectric focusing

COS-1 cells were transfected in the presence of [^{35}S]methionine during an 8 h pulse period. To analyse enzyme activity in culture medium, cells had to be grown without serum. Isoelectric focusing was performed in focusing columns with a gradient from pH 3 to 10 stabilized in a 0–35% (w/v) sucrose gradient as described previously (Quintern et al., 1989a).

SDS/PAGE and autoradiography

PAGE was carried out under reducing conditions using 0.75 mm slab gels containing 10% acrylamide. The gels were fixed in 10% acetic acid/20% propan-2-ol for 30 min and impregnated with Amplify (Amersham) before vacuum drying. Fluorography was performed using Kodak X-Omat AR films.

Immunofluorescence studies

Some 24 h after transfection, the COS-1 cells were trypsinized and plated on to coverslips. After further incubation for 12 h, cells were fixed with acetone for 10 min at −70 °C, washed thoroughly with PBS and blocked with 3% ovalbumin/TBS for 60 min at 37 °C. After further washing with 0.1% ovalbumin/TBS, the fixed cells were incubated with a 1:200 dilution of the rabbit anti-ASM serum for 30 min at 4 °C. Incubation with DTAF-labelled purified goat Fab′ fragments was performed for 60 min at 4 °C. A Zeiss Axiovert 35 was used for fluorescence microscopy.

RESULTS

In order to analyse the biosynthesis of ASM, specific high-affinity antibodies were raised against carbohydrate-free recombinant enzyme protein from *E. coli*, which were transformed with appropriate prokaryotic expression vectors. These plasmids encoded most of the ASM cDNA, except the portion encoding the potential signal peptide and additional 5′-sequences responsible for expression of a fusion protein containing an N-terminal hexahistidine-affinity tail. Recombinant protein was purified by nickel-ion chelating chromatography and further used as antigen for antibody preparation. Immunization of Chinchilla bastard rabbits yielded antiserum preparations suitable for precipitation of more than 90% of ASM activity from crude fibroblast homogenate as well as purified enzyme from various sources, contaminating hydrolase activities remaining in the supernatant. In contrast with previous reports, precipitation of ASM was not influenced by the addition of non-ionic detergents (0.5–2.0% Nonidet P20).

Initial biosynthesis studies on COS-1 cells transfected by wild-type cDNA revealed a more complex protein pattern than expected. After 1 h of pulse labelling with [^{35}S]methionine, immunoprecipitation of ASM from total cell extracts and fluorography of proteins separated by SDS/PAGE revealed three major translation products: polypeptides of apparent molecular mass 75 and 72 kDa, usually detected as a single diffuse band, and 57 kDa (Figure 1). Deglycosylation of these proteins with β-endo-*N*-acetylglucosaminidase F (Endo F) resulted in sharp bands of reduced size (64, 61 and 47 kDa respectively). It is worth mentioning that the amount of 57 kDa component varied significantly between 5 and 20% of total cellular immunoreactive material in different experiments. The culture medium of transiently transfected COS-1 cells, which are known to secrete substantial quantities of recombinant protein as a result of the enormous overexpression of transfected DNA, yielded only the two predominant ASM-derived forms (approx. 75 and 57 kDa in size) (Figure 1). However, deglycosylation experiments indicated that exported 75 kDa protein consists of a 61 kDa protein core identical in size with that of the 72 kDa form of the cell homogenate. The increase in relative molecular mass of the secreted protein may be caused by additional carbohydrate processing. As cellular 75 kDa ASM (with a polypeptide core of

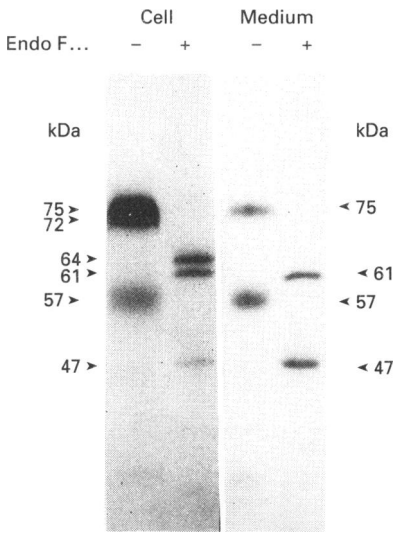

Figure 1 Processing and N-glycosylation of ASM expressed in COS-1 cells

COS-1 cells transfected with wild-type cDNA (wt ASM) were pulse labelled with [^{35}S]methionine for 1 h. For immunoprecipitation of culture medium, COS-1 cells were chased for a further 4 h period. ASM was immunoprecipitated with anti-ASM serum and treated with (+) or without (−) EndoF.

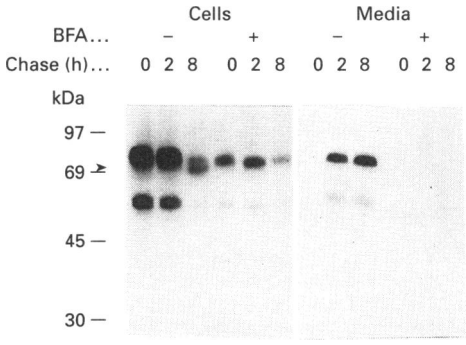

Figure 2 Pulse–chase analysis of ASM from transfected COS-1 cells in the presence of brefeldin A

Wild-type transfected COS-1 cells were labelled with [^{35}S]methionine for 1 h and subsequently chased for the times indicated (−). Experiments using brefeldin A (BFA) (+) were performed as described in the Experimental section. The arrow indicates the mature lysosomal form of ASM (70 kDa).

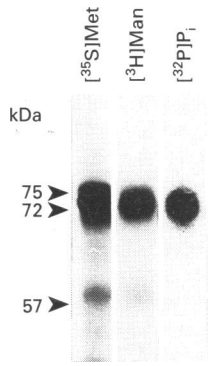

Figure 3 Metabolic labelling of transfected COS-1 cells with [^{35}S]methionine, [^{3}H]mannose or [^{32}P]P_i

Labelling was carried out during a 4 h period and cell homogenate was separated by SDS/PAGE as described in the Experimental section.

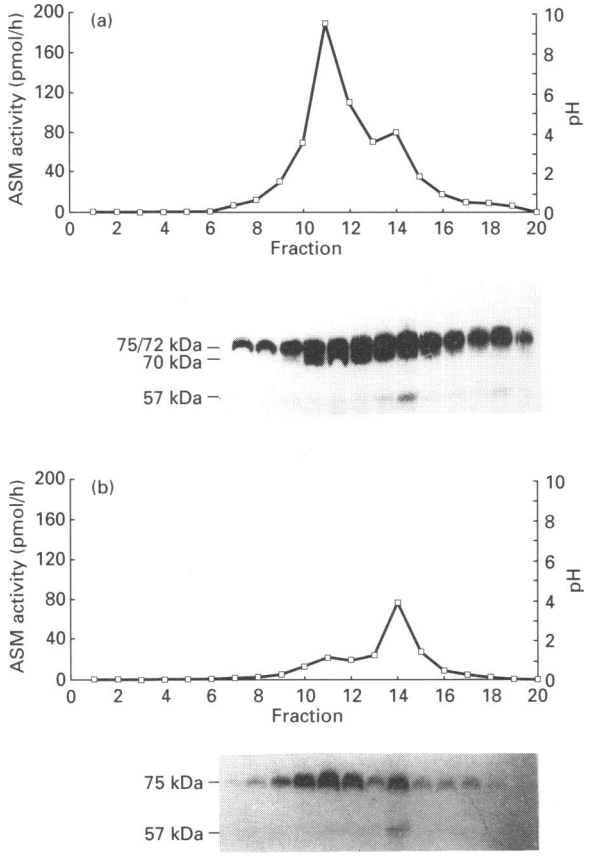

Figure 4 Isoelectric focusing

Cell extracts (**a**) and serum-free media (**b**) from COS-1 cells wild-type cDNA transfected in the presence of [^{35}S]methionine over an 8 h period were subjected to isoelectric focusing as described in the Experimental section. Each fraction was used to determine ASM activity (□) using [^{3}H]choline-labelled sphingomyelin as substrate and for immunoprecipitation experiments in parallel.

64 kDa) is obviously not detected in the culture medium, this polypeptide is presumably exclusively located inside the ER and may consist of the ASM precursor still linked to the putative signal peptide with a theoretical mass of about 4 kDa. Although we believe that the above is the most likely interpretation of our observations, a word of caution is required in that the possibility of the intracellular 75 kDa form also generating some 61 kDa form cannot be rigorously excluded from our experiments. However, the proportion of the 61 kDa form derived from the 75 kDa form, if it exists at all, must be very small.

Pulse–chase studies on transfected COS-1 cells indicated that further processing of the 72 kDa ASM precursor resulted in the formation of the mature form of ASM with an apparent molecular mass of 70 kDa over a 6–8 h chase period, whereas the 57 kDa form was not subjected to significant processing steps but was lost after about a 6 h chase (Figure 2). Surprisingly, processing was not dependent on any detectable proteolytic event as judged by deglycosylation experiments (K. Ferlinz, R. Hurwitz, G. Vielhaber, K. Suzuki and Konrad Sandhoff, unpublished work). Additional experiments using brefeldin A to capture newly synthesized protein inside the ER/Golgi apparatus strongly suggest that maturation of ASM precursor to the 70 kDa form is restricted to the acidic environment of endosomal/lysosomal compartments (Figure 2). The ASM-cDNA-derived 57 kDa protein was monitored independently of blocked vesicular trafficking. As expected, secretion of this protein was completely inhibited after addition of the drug.

Biosynthetic labelling of transfected COS-1 cells with [^{3}H]mannose or [^{32}P]P_i provided evidence that only the high-molecular-mass forms of ASM carry N-linked oligomannosyl phosphate residues, known to be responsible for regular sorting for the lysosomal pathway. As shown in Figure 3, the 57 kDa form of ASM lacked detectable amounts of mannose-linked phosphate residues.

To assess further the potential physiological relevance of different ASM components, we analysed them for enzyme activity. The cell homogenate and medium of COS-1 cells transfected with either wild-type cDNA or the vector only and cultured in the presence of [^{35}S]methionine were subjected to isoelectric focusing. Fractions of different pH were immunoprecipitated and assayed for enzyme activity in parallel (Figure 4). Most ASM activity in the homogenate could be assigned to fractions containing proteins of isoelectric point (pI) 6.8–7.2, whereas a minor portion comprising about 10% of total enzyme activity peaked around pI 5.0. Immunoreactive material from cell-homogenate-derived isoelectric-focusing fractions was separated by SDS/PAGE and further visualized by fluorography (Figure 4a). Correlation of catalytic activity with precipitable proteins suggests that mature lysosomal ASM (70 kDa) was restricted to fractions of pH 6.8–7.2. The major proportion of the 57 kDa form could be isolated from fractions of pH 4.8–5.0. Interestingly, ASM precursor (72 kDa) which was spread over the entire pH range (3–8) did not coincide with distinct fractions of ASM activity, implying that this protein exhibits low, if any, enzyme activity. Analysis of the corresponding medium revealed two molecular forms of ASM, the secretory form of the ASM precursor (75 kDa) and the small 57 kDa form (Figure 4b). Determination of activity revealed some, but low, sphingomyelin degradation in fractions of pH 4.8–5.0 which again correlated with a faint band of 57 kDa ASM. The medium from mock-transfected COS-1 cells did not show any ASM activity.

As both enzymically active forms of ASM are of potential physiological significance, the question arose whether 57 kDa ASM originates from early proteolytic cleavage of a common precursor or as the result of different translation-initiation sites of the corresponding gene. Therefore we analysed the functionality of the two possible in-frame initiation codons (AUGs) as well as the DNA region encoding the potential ER import signal sequence responsible for biosynthesis and proper targeting

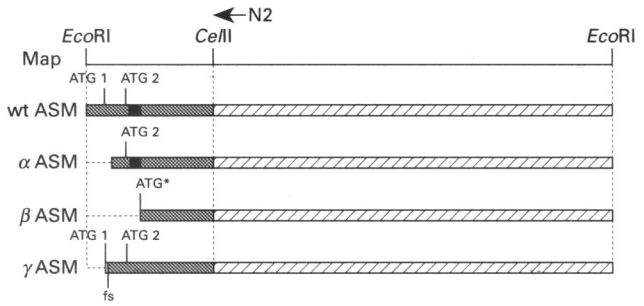

Figure 5 Schematic illustration of wild-type cDNA and 5′-deletion mutants

The map shown at the top indicates the location of restriction enzymes used for construction of the mutants. The filled boxes depict the area encoding the signal sequence. DNA portions illustrated as shaded bars represent PCR-derived mutagenized cDNA sequences and crossed regions are derived from regular wild-type (wt) cDNA clones. ATG* indicates an artificially inserted initiation codon to achieve expression of β ASM and fs indicates the position of the frameshift insertion of γ ASM. N2 indicates the position of the antisense primer used for mutagenic PCR amplification. Sense primers are located at the 5′ end of the respective constructs.

Table 2 Transient expression of ASM in COS-1 cells

Values are means of three independent transfection experiments using different batches of plasmid DNA. Enzyme activities were determined by incubating crude cell homogenate with [^{3}H]choline-labelled sphingomyelin.

Construct	ASM activity (nmol/h per mg)
COS-1 cells	8.8±1.0
p91023(B)	9.0±1.3
wt ASM-i (antisense)	7.7±0.5
wt ASM (sense)	62.2±3.1
α ASM	64.0±4.0
β ASM	10.2±0.8
γ ASM	12.8±1.5

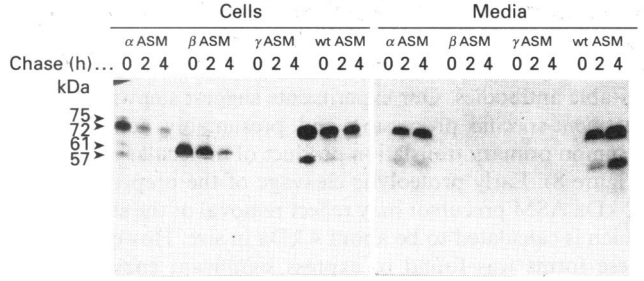

Figure 6 Pulse–chase analysis of ASM in COS-1 cells expressing normal and mutant cDNAs

COS-1 cells were transfected with wild-type cDNA (wt ASM) and mutant cDNAs (α ASM, β ASM, γ ASM). After transfection, cells were labelled with [^{35}S]methionine for 1 h and chased for the times indicated.

by expressing human ASM cDNA (wt ASM) mutagenized at appropriate sites (Figure 5).

Enzymic studies on COS-1 cells transfected with either wild-type or mutant cDNAs indicated that wild-type cDNA (wt ASM) and α ASM, which lacks the first ATG, expressed enzymically active proteins to a similar extent (Table 2). Insertion of a frameshift between the two potential initiation codons (γ ASM) did not result in elevated levels of ASM activity compared with mock-transfected cells. Similarly to γ ASM expression, the mutant cDNA lacking the region encoding the signal peptide (β ASM) also failed to produce catalytically active ASM (Table 2).

Metabolic labelling of the transfectants followed by immunoprecipitation was performed to visualize the presence of cross-reactive material derived from the various mutagenized cDNA constructs (Figure 6). COS-1 cells transfected with either wild-type ASM or α ASM synthesized almost identical amounts of ASM precursor as well as the 57 kDa form. Transfection with γ ASM failed to induce expression of significant amounts of immunoreactive protein, implying that the translation product from the first AUG after introduction of a frameshift mutation has no sequence similarity with the wild-type protein and that the

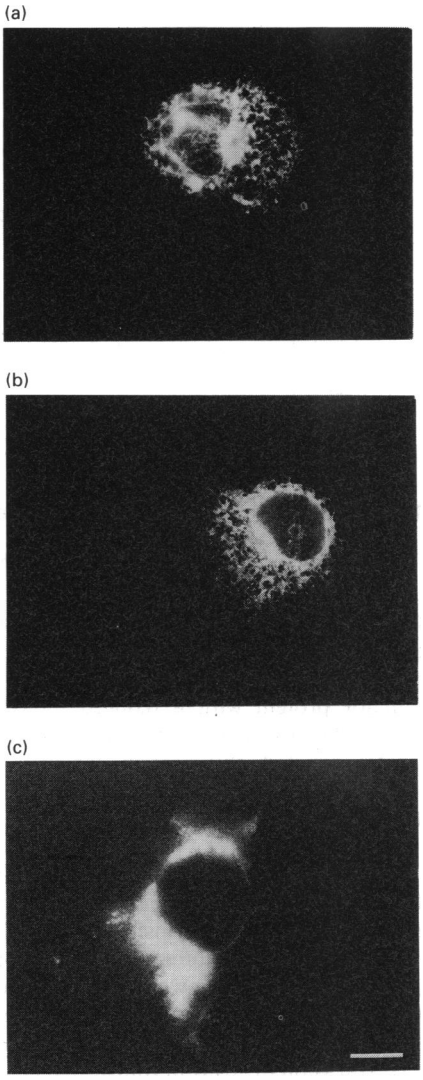

Figure 7 Subcellular localization of normal and mutant ASM by indirect immunofluorescence

Fluorescence micrographs of COS-1 cells transfected with wild-type cDNA construct (**a**), α ASM (**b**) and β ASM (**c**). The results for γ-ASM-transfected COS-1 cells are not presented because of the absence of immunocross-reactive material (Bar 10 μm.)

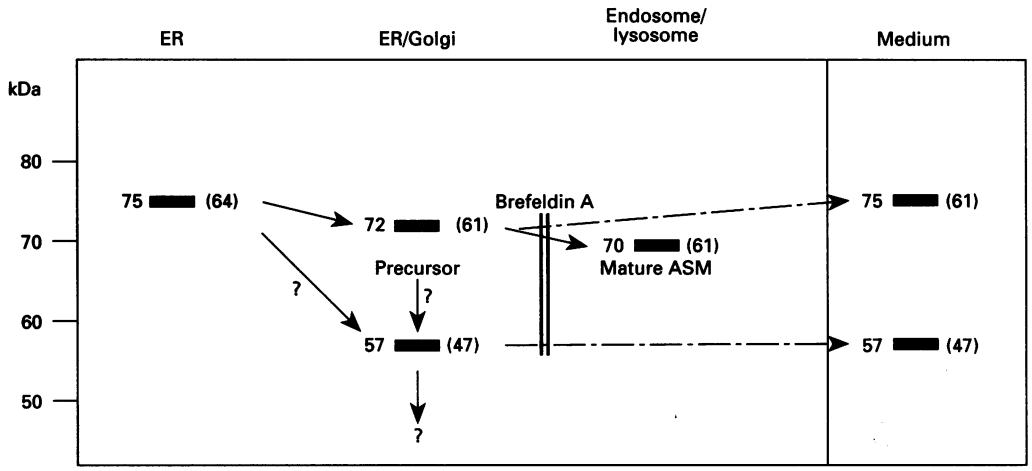

Figure 8 Schematic illustration of compartment-specific processing of ASM

Numbers refer to apparent molecular masses (kDa) of the proteins as judged by SDS/PAGE. Numbers in parentheses indicate the protein size after deglycosylation with Endo F.

second AUG is not used for translation when the first AUG is present (Figure 6). In the absence of the first AUG, the second AUG can be an effective initiator.

Only one major protein with a reduced molecular size of approximately 60 kDa was detected by the antibodies in β ASM transfectants. As expected, this protein, which is missing the signal peptide, is not imported into the ER lumen; it is not glycosylated and thus cannot be transported by a regular vesicular mechanism (Figure 6).

To investigate further whether peptides derived from mutagenized vector constructs are transported and distributed similarly to the wild-type protein, transfected COS-1 cells were analysed by indirect immunofluorescence microscopy. Normal and α ASM-derived polypeptides were detected throughout the vacuolar apparatus of the cell. Strong fluorescence staining of compartments representing ER and Golgi is presumably due to enormous overexpression of ASM in transfected cells (Figure 7). The γ-ASM-transfected COS cells completely failed to express cross-reactive protein, whereas fluorescence staining of β ASM transfectants revealed fluorescence exclusively in the cytosol, indicating the pivotal role of the potential signal sequence for luminal ER translocation.

DISCUSSION

As production of mono- and poly-clonal antisera raised against native human ASM from various tissues by several groups failed in terms of specificity of the resulting immunoglobulins (Freeman et al., 1984; Weitz et al., 1985; Maehira and Takaesu, 1987; Rousson et al., 1987), we decided to overproduce in *E. coli* a carbohydrate-free recombinant polypeptide chain containing the entire protein core except the potential signal peptide. Standard immunization of rabbits finally led to the first specific polyclonal anti-ASM antibody preparation reported so far which is insensitive to high concentrations of non-ionic detergent. The antiserum specificity recognizes native human enzyme and is capable of precipitating more than 90% of total ASM activity from crude cell extracts of human tissues. The 10% remaining may be the result of unfavourable protein conformations, masking of the polypeptide chain by N-glycosylation at the six potentially available sites of the native enzyme or aggregate formation.

Initial biosynthesis studies on human fibroblasts revealed a complex protein pattern consisting of several components of different size. As the pure human urinary enzyme has been exclusively characterized as a glycosylated protein of about 70 kDa, the presence of further protein components was not expected. However, these data support previous reports considering two distinct co-purified ASM-related proteins, a 72 kDa and 54–57 kDa component of undefined origin, which were detected by Western-blot analysis using monoclonal antibodies against protein derived from human placenta (Rousson et al., 1993). Analysis of ASM from cDNA-transfected COS-1 cells, performed to verify the specificity of the antiserum, resulted in detection of proteins similar in size to those from cultured fibroblasts, indicating that they originate from expression of a single mRNA species. In order to analyse the functions of these forms, COS-1 cells were wild-type and mock-transfected in the presence of [^{35}S]methionine and cell homogenate as well as media were separated by isoelectric focusing. It should be noted that ASM activity from crude cell extracts was found earlier to peak at at least two distinct isoelectric points, comparable to those in our studies (Callahan et al., 1978; Rousson et al., 1983; Quintern et al., 1989a). So far, correlation of these data with distinct processing forms of ASM have failed because of the lack of suitable antibodies. Our experiments suggest stepwise and compartment-specific processing and presumably activation of a common primary translation product of molecular mass 75 kDa (Figure 8). Early proteolytic cleavage of the preproform into a 72 kDa ASM precursor may reflect removal of the signal peptide which is calculated to be about 4 kDa in size. However, none of these forms was found to express significant enzyme activity. Similar observations were made in studies with cultured I-cell fibroblasts that lacked regular lysosomal sorting by a mannose-6-phosphate-mediated pathway. Although they were shown to secrete most of the newly synthesized ASM precursor, enzyme activity in the respective culture medium was only slightly increased compared with that in control cells (Klaus Ferlinz, R. Hurwitz, G. Vielhaber, K. Suzuki and K. Sandhoff, unpublished work). Biosynthesis and targeting of a catalytically inactive ASM precursor may also prevent the early release of the second messenger, ceramide, during transport of the enzyme. Processing in the acidic compartments of the cell finally yields the prevalent mature ASM 70 kDa form. Interestingly, this maturation is not brought about by detectable proteolytic processing as

judged from deglycosylation experiments. Thus modification of distinct carbohydrate moieties, alteration in putative protein phosphorylation or maturation by removal of only a few N-terminal amino acids, as described for maturation of the β-chain of hexosaminidase (Quon et al., 1989), may be the cause of activation of the catalytic properties. A further 57 kDa protein component which also contributes limited ASM activity already occurs inside the ER/Golgi complex. During several experiments, formation of this form was very variable compared with total immunoreactive material (5–20%). The reason for this possibly regulated early activation of ASM is not yet understood but cell culture conditions, i.e. serum content, pH or time of transient expression, may be involved. Experiments using brefeldin A, which specifically blocks regular vesicular transport from ER to Golgi (Klausner et al., 1992), had no influence on the amount of 57 kDa protein formed but clearly indicates that maturation of 70 kDa ASM is dependent on the acidic environment. Further labelling experiments with [^{3}H]mannose and [^{32}P]P_i gave rise to different targeting of the two active ASM forms. The 57 kDa form, which does not carry oligomannosyl phosphate residues in contrast with the high-molecular-mass forms of ASM, is presumably not targeted via the mannose-6-phosphate-mediated pathway. Thus it is questionable whether this enzymically active protein is destined for the lysosomes or whether it may have different functions.

In order to verify that the 57 kDa ASM is a product of early proteolytic cleavage and is not the result of the use of different translation-initiation sites on the corresponding gene, ASM cDNA was mutagenized at appropriate sites. Translation of alternatively spliced mRNAs could be excluded, as studies were performed using wild-type cDNA constructs (Schuchman et al., 1991).

The theory of translation initiation of proteins implies that initiation is almost exclusively mediated by the AUG codon located most 5′ on the mRNA. In this 'scanning model', the 40S ribosome, together with other translation-initiation factors, binds to the capped terminus of the mRNA and advances until the first AUG is encountered (Kozak, 1989). Furthermore, the nucleotides surrounding the AUG of interest correlate with the efficiency of translation initiation and finally commit the ribosomes to initiate peptide synthesis and elongation until a stop codon is reached. Thus expression of two or more polypeptides originating from different initiation codons usually requires different 5′-truncated mRNA forms which might be generated by promoter switching or alternative splicing of common heterogeneous nRNAs.

Northern-blot analysis as well as primer-extension experiments on the mRNA of ASM provided evidence that the cell transcripts are close in size to that of the full-length cDNA sequence. The majority of mRNA which is 5′-extended for about 100 bases of known cDNA does not contain any further AUG (K. Ferlinz, R. Hurwitz, G. Vielhaber, K. Suzuki and K. Sandhoff, unpublished work). These experiments suggest an untranslated 5′-sequence of about 180 bp which is in agreement with sequences expected from vertebrate mRNAs (Kozak, 1987).

Our experiments demonstrate that the first of two potential in-frame AUGs upstream of the signal-sequence-encoding portion is exclusively used for efficient translation initiation *in vivo* and that the use of any other potential downstream AUGs can be excluded. Direct evidence confirming this fact came from the transfection of cDNA constructs containing a single base insertion between the two potential initiation codons. Translation of the corresponding mRNAs in transfected COS cells failed to produce enzymically active and immunoreactive peptides. We suggest that protein biosynthesis started solely from the first AUG in the mutant mRNA and proceeded through the frame-shift insertion until a stop codon prevented further translation. This presumably resulted in a peptide of 48 amino acid residues in length with obviously no sequence similarity to the native polypeptide and therefore not immunoprecipitated by the antiserum. Results similar to those for ASM were obtained for the β-chain of hexosaminidases and β-glucocerebrosidase, for which only the first in-frame AUG was utilized for translation initiation *in vivo* (Sorge et al., 1987; Sonderfeld-Fresko and Proia, 1988; Neote et al., 1990). Biosynthesis and processing of recombinant sphingomyelinase starting from the second potential AUG, which we presume to be identical with that in the wild-type protein, indicates that even the loss of a functional primary initiation codon would not result in pathological enzyme deficiency.

A signal sequence responsible for ER import of nascent ASM polypeptides which comprises the N-terminus of the propeptide was postulated by hydrophobicity and secondary-structure analysis of cDNA data (Schuchman et al., 1991) as outlined by Garnier et al. (1978). The authenticity of the predicted sequence was evaluated by expression of the respective cDNA constructs. Proteins lacking the signal sequence were shown to be located cytosolically because transduction into the ER lumen was prevented.

The data presented here clearly establish that the three forms of ASM are generated from the same nascent polypeptide derived from a single processed transcript. These data alone, however, cannot define the position of the 57 kDa form within the 75 kDa form nor can they prove that the 72 kDa form results from removal of the signal peptide from the 75 kDa form. The precise information would require sequencing of all these forms. However, we do have data to indicate that the 57 kDa form is generated largely by cleavage of the N-terminus of the 75 kDa form. When the first two glycosylation sites were eliminated by site-directed mutagenesis and the protein expressed, the normal 75 and 72 kDa forms decreased to sizes expected if the two carbohydrate chains were missing whereas the size of the 57 kDa form remained unchanged (K. Ferlinz, R. Hurwitz, G. Vielhaber, K. Suzuki and K. Sandhoff, unpublished work). This observation clearly indicates that the 57 kDa form does not include the first two glycosylation sites. If the N-terminus is cleaved at or immediately after the second glycosylation site, the remainder should have a sugar-free size of approximately 50 kDa. As there are four glycosylation sites within the remaining C-terminus, this segment will generate a protein of 57–58 kDa when fully glycosylated. Thus the N-terminus of the 57 kDa form must be close to the second glycosylation site and, if C-terminus trimming is involved at all, it cannot be extensive. This information also makes it possible to generate the large-form-specific antibody by using the segment of the larger forms between the N-terminus and the second glycosylation site.

In conclusion, processing of human ASM precursor, which expresses low, if any, catalytic activity, results in two distinct polypeptides. One of these is already generated by proteolytic cleavage inside the ER/Golgi complex whereas the predominant mature 70 kDa form is exclusively processed after sorting for the endosomal/lysosomal pathway. At present it is not clear whether both forms of mature ASM finally reach the lysosomal compartments or whether they have distinct biochemical functions. The fact that the 57 kDa protein obviously does not contain oligomannosyl-linked phosphate residues may point to a different method of sorting, as expected for regular soluble lysosomal proteins. Furthermore, detection of variable relative amounts of cellular 57 kDa ASM implies that activation of the enzyme early on inside the ER and Golgi compartments may be achieved by certain regulated proteases which trigger early processing events.

We thank H. Moczall and C. Klein for expert technical assistance. This work was supported by the Deutsche Forschungsgesellschaft, DFG grant 257/19-1.

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Received 27 September 1993/24 January 1994; accepted 1 March 1994

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