# Functional studies of human intestinal alkaline sphingomyelinase by deglycosylation and mutagenesis

Jun WU*, Gert H. HANSEN†, Åke NILSSON* and Rui-Dong DUAN*[1]

*Gastroenterology Lab, Biomedical Center B11, Lund University, S-221 84 Lund, Sweden, and †Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, DK2200, Copenhagen, Denmark

Intestinal alk-SMase (alkaline sphingomyelinase) is an ectoenzyme related to the NPP (nucleotide phosphodiesterase) family. It has five potential N-glycosylation sites and predicated transmembrane domains at both the N- and C-termini. The amino acid residues forming the two metal-binding sites in NPP are conserved, and those of the active core are modified. We examined the functional changes of the enzyme induced by deglycosylation and mutagenesis. Treating alk-SMase cDNA-transfected COS-7 cells with tunicamycin rendered the expressed enzyme completely inactive. Mutations of the five potential N-glycosylation sites individually and in combination showed that these sites were all glycosylated and deficient glycosylation decreased the enzyme activity. Immunogold labelling showed that the wild-type enzyme was mainly located in the plasma membrane, whereas the C-terminal domain-truncated enzyme was released into the medium. Deglycosylation blocked the release of the enzyme that accumulated in endosome-like structures. The enzyme activity was also decreased by mutations of the residues forming the putative metal-binding sites and the active core. Substitution of the active core sequence with that of NPP or mutation of T75 in the core abolished the enzyme activity against sphingomyelin but failed to render the enzyme NPP active. Our results indicate that alk-SMase activity is severely affected by defective N-glycosylation and structural alterations of the putative metal-binding sites and the predicted active core.

Key words: alkaline sphingomyelinase, cancer, glycosylation, mutation, nucleotide phosphodiesterase.

## INTRODUCTION

Intestinal alk-SMase (alkaline sphingomyelinase) was discovered more than 30 years ago [1]. The enzyme catalyses the hydrolysis of exogenous and endogenous SM (sphingomyelin) in the gut and generates multiple lipid messengers such as ceramide, sphingosine and sphingosine-1-phosphate, which have important roles in regulating cell proliferation, differentiation and apoptosis [2]. Evidence is accumulating that digestion of SM in the gut may have specific implications in colonic tumorigenesis [3,4]. Supplementing SM and ceramide analogues in the diet could inhibit the formation of colonic aberrant crypt foci and colon cancer in mice treated with 1,2-dimethylhydrazine [5–7]. We reported previously that the pathogenesis of colorectal adenocarcinomas and longstanding ulcerative colitis is associated with a decrease in alk-SMase activity [8–10]. Additionally, SM metabolism in the gut may affect the development of atherosclerosis. Increasing the amount of SM in the intestine or in the cell culture medium was found to inhibit the absorption of cholesterol of enterocytes [11,12]. Ceramide hydrolysis in the gut may also influence the intestinal production of lipoproteins [13].

We have purified alk-SMase [14–16], identified the gene and cloned the cDNA [17]. Sequence analysis and immunogold labelling studies showed that the enzyme is an ectoenzyme located on the surface of the microvillar membrane [16,17]. The putative structure of human alk-SMase is shown in Figure 1. The enzyme has 458 amino acid residues and TMpred analysis indicates the presence of transmembrane domains at both the N- and C- termini [17]. The N-terminal domain is a predicted signal peptide and the C-terminal domain is a signal anchor. We recently showed that truncation of the C-terminal anchor resulted in release of the enzyme into the cell culture medium in an active form [18]. The enzyme has five potential N-glycosylation sites. Whether these sites are glycosylated and whether the glycosylation is of importance for the function and translocation of the enzyme are currently unknown. Sequence searching of the database showed that alk-SMase shares no similarity with either acid or neutral SMase but is related to the NPP (nucleotide phosphodiesterase) family. The amino acids forming two metal co-ordinating sites of NPP are conserved, but the sequence of the predicted active core has been modified in alk-SMase [17]. The importance of these sites for the activity and substrate specificity of alk-SMase, which was found not to have NPP activity [17], has not been examined.

In the present study, we addressed the importance of glycosylation and the predicted active core, the putative metal-binding sites and the C-terminal anchor of alk-SMase for function, secretion and substrate specificity.

## MATERIALS AND METHODS

### Materials

COS-7 cells were purchased from A.T.C.C. SM was purified from bovine milk and labelled with [$^{14}$C-CH$_3$]choline ([$^{14}$C]SM). The specific activity of the labelled SM was 56 $\mu$Ci/mg. Plasmid pCDNA4/TO/Myc-His B, LIPOFECTAMINE™ 2000, AccuPrime™ pfX DNA polymerase, and the primers used were purchased from Invitrogen (Paisley, U.K.). GFX™ DNA and gel band purification kit were obtained from Amersham Biosciences (Uppsala, Sweden). Tunicamycin, cell culture medium and other

Abbreviations used: alk-SMase, alkaline sphingomyelinase; NPP, nucleotide phosphodiesterase; pNPP, *p*-nitrophenyl phenyl phosphate; pNTMP, *p*-nitrophenyl thymidine 5′-monophosphate; SM, sphingomyelin; for brevity, the single-letter system for amino acids has been used, P441, for example, means Pro$^{441}$.

[1] To whom correspondence should be addressed (email Rui-dong.duan@med.lu.se).

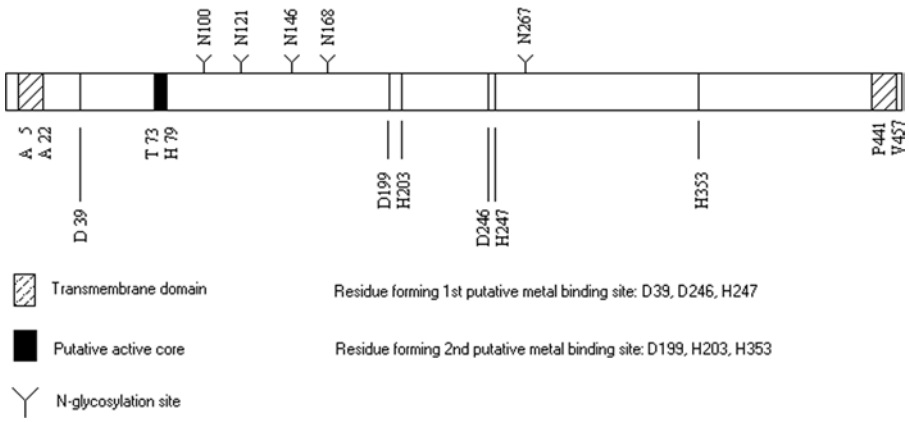


**Figure 1 Schematic representation of the putative structure of human alk-SMase**

Transmembrane domains at both the N- and C-terminal, the potential glycosylation sites, the modified activity core and the conserved amino acids forming the metal-binding sites in NPP are indicated.

chemical agents used were purchased from Sigma (Stockholm, Sweden).

### SMase assay

Alk-SMase activity was determined by a method described previously [19]. Briefly, samples (5 $\mu$l) were mixed with 95 $\mu$l of 50 mM Tris/HCl buffer (pH 9.0) containing 0.15 M NaCl, 2 mM EDTA, 6 mM taurocholate (assay buffer) and 0.80 $\mu$M [$^{14}$C]SM ($\sim$8000 d.p.m.) and incubated at 37 °C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2:1, v/v) followed by centrifugation at 8500 ***g*** for 10 s. An aliquot (100 $\mu$l) of upper phase containing the cleaved phosphocholine was analysed for radioactivity by liquid-scintillation counting.

### Transient expression of alk-SMase in COS-7 cells

Transient expression of alk-SMase in COS-7 cells was performed as described in [17]. The COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10 % (v/v) heat-inactivated fetal calf serum and 2 mM glutamine to approx. 90 % confluence. The cells were then transfected with 4 $\mu$g of constructed plasmid with wild-type or mutated alk-SMase cDNA insert in the presence of LIPOFECTAMINE™ 2000, followed by incubation of the cells for 48 h. The medium was collected and the cells were scraped and lysed in 50 mM Tris/HCl buffer (pH 7.4) containing 1 mM PMSF, 2 mM EDTA, 0.5 mM dithiothreitol, 10 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin and 10 mM taurocholate, followed by sonication for 10 s. After centrifugation at 12000 ***g*** for 10 min at 4 °C, the cell-free extract was collected.

### Effects of glycosylation on the function of alk-SMase

The effects of glycosylation on the function of alk-SMase were studied by inhibition of glycosylation and by mutation of glycosylation sites. First, we transfected COS-7 cells with wild-type alk-SMase cDNA. Tunicamycin was added to the culture medium, 6 h after transfection, to a final concentration of 10 $\mu$g/ml to inhibit glycosylation, followed by incubation for an additional 42 h. At the end of incubation, the cell lysate was prepared and alk-SMase activity in the lysate was determined. Secondly, we mutated the five potential N-glycosylation sites individually and in various combinations, followed by expression of the mutated cDNA in COS-7 cells for 48 h. The functional changes in alk-SMase induced by the mutations were determined. To study the effect of deglycosylation on transport of alk-SMase in cells, mutation of all the glycosylation sites was performed in C-terminal-truncated enzyme, since we previously found that the C-terminal-truncated alk-SMase can be transported to the plasma membrane and released into the culture medium [18]. Alteration of the enzyme released into the medium was examined by assaying alk-SMase activity, and alteration of cellular location of the enzyme induced by deglycosylation was examined by immunogold labelling.

### Western blotting of alk-SMase

The wild-type and the mutated alk-SMase cDNAs were subcloned into the expression vector pcDNA4/TO/Myc-His B. After expression, 75 $\mu$g of cellular proteins in the lysate or 10 $\mu$l of the cell culture medium was subjected to SDS/PAGE (10 % gel) and transferred on to a nitrocellulose membrane. The membrane was probed with an anti-Myc antibody (1:5000) for 2 h. After washing, the membranes were allowed to react with an anti-mouse IgG antibody conjugated with horseradish peroxidase for 1 h. The alk-SMase bands were identified by ECL® advance reagents and the re-emitted light was recorded on a Kodak X-ray film. The whole procedure was performed according to the instructions of the manufacturer.

### C-terminus truncation of alk-SMase

Expression of two types of C-terminal-truncated alk-SMases was performed. In the first experiment, the cDNA encoding a type of alk-SMase having the C-terminal anchor from P441 to A458 truncated was amplified by PCR, using the sense primer 5′-tcggtaccgaaagcatgagaggcccggccgtcctc-3′ and the antisense primer 5′-tagcggccgcccctgctgctgggcgggag-3′. The second experiment was designed to truncate the C-terminus from E416 to A458, which is coded by the last translated exon (exon 5). The sense primer was the same as the one described above and the antisense primer was 5′-tagcggccgcctgtgtgcagcatgggcagc-3′. The plasmid pcDNA4/TO/Myc-His B with full-length SMase cDNA insert was used as a template. The PCR products were isolated on 1 % agarose gel, purified, digested with *Kpn*I–*Xba*I and inserted into *Kpn*I and *Xba*I sites of the plasmid. The constructed cDNAs and the wild-type alk-SMase cDNA were transiently expressed in COS-7 cells. The alk-SMase activities in both cell-free extracts and cell culture medium were determined and the expressed enzyme was examined by Western blotting.

**Table 1 Sequences of the primers used in the present study**

| Mutation | Primer |
|---|---|
| M74K (antisense) | 5′-GGTGAAGTGGCAGGGGCTGGTCTTGGTGACAAAGGCGGGGGTCA-3′ |
| T75A (antisense) | 5′-CAGGGTGAAGTGGCAGGGGCTGGCCATGGTGACAAAGGCGGGGGT-3′ |
| S76F (antisense) | 5′-ACCAGGGTGAAGTGGCAGGGGAAGGTCATGGTGACAAAGGCGG-3′ |
| C78N (antisense) | 5′-GGTGACCAGGGTGAAGTGGTTGGGGCTGGTCATGGTGACAA-3′ |
| D199A (antisense) | 5′-CGTACCTGTGGCCCGTGGAGGCCGGCTCCCCGAAGTAGAGTG-3′ |
| 5D246A (sense) | 5′-GCCTCAACCTGATCATCACATCCGCCCACGGCATGACGACCGTGGACAAAC-3′ |
| N100Q (antisense) | 5′-GCAGCTTCACCTTGCTGGTGGTTTGGTAGTACATGTTGTGAACCACC-3′ |
| N121Q (antisense) | 5′-TGATCCAGATGGGCACGCTGCCTTGGTCCCACCACCTCTGGATGCCC-3′ |
| N168Q (antisense) | 5′-TGTTCGCTCTCCACTCCGTCTCTTGTTTGTAGTTGTGTGCGATGCCT-3′ |
| N146Q (antisense) | 5′-CAGCCACCCCTTGGTAGGTGACTTGCCCGCCCGGGTAGAAGAAGGA-3′ |
| N267Q (sense) | 5′-GGTTGAATTCCACAAGTTCCCCCAATTCACCTTCCGGGACATCGAGT-3′ |

### Site-directed mutation

Site-directed mutagenesis was performed by the megaprimer PCR method [20]. The oligonucleotides used for mutating the predicted active core [M74K ($Met^{74} \rightarrow Lys$), T75A, S76F and C78N], the two predicted metal co-ordinating sites (D199A and F246A) and the five potential N-glycosylation sites (N100Q, N121Q, N146Q, N168Q and N267Q) are shown in Table 1, in which the altered codons are underlined. The sense and antisense primers were 5′-tcggtaccgaaagcatgagaggcccggccgtcctc-3 and 5′-tagcggccgcctgcgacctcagacagaagaat-3′, with the wild-type alk-SMase cDNA used as a template. The mutated genes were cloned into the vector pcDNA4/TO/myc-His B at *Kpn*I and *Not*I sites as the wild-type gene [17].

N100/121Q two-site mutations were performed similarly, using the N100Q mutated alk-SMase gene as a template and N121Q (antisense) primer as the mutagenesis primer. Combinations of 3, 4 and 5 site mutations of N100/121/168Q, N100/121/168/146Q, N100/121/168/146/267Q were performed in a stepwise manner. Effects of N-glycosylation on the activity and transport of alk-SMase were studied in COS-7 cells transfected with either wild-type, R440-truncated or T415-truncated alk-SMase as described above. After a 48 h incubation, the activities of alk-SMase from both the wild-type enzyme and the truncated one were determined in both cell culture medium and cell lysate, and the proteins were examined by Western blotting. To study the alteration of alk-SMase transport induced by deglycosylation, COS-7 cells were transfected with wild-type C-terminal-truncated enzyme and the C-terminal-truncated enzyme with five glycosylation sites mutated. After incubation for 48 h, the enzyme activities in the cell lysate and culture medium were determined and the enzyme protein was analysed by Western blotting.

### Immunogold electron microscopy

Gold particles (7 nm) were prepared by the method of Slot and Geuze [21] and conjugated with goat anti-rabbit IgG [22]. COS-7 cells were transfected with wild-type C-terminal-truncated alk-SMase and the C-terminal-truncated alk-SMase with all N-glycosylation sites mutated, for 48 h. The cells were washed with PBS buffer and fixed in 2 % (w/v) paraformaldehyde/0.1 % glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) for 30 min at 4 °C. After a wash, the cells were carefully detached from the culture dish by a rubber policeman, collected in an Eppendorf tube and stored in 1 % paraformaldehyde at 4 °C. The cells were transferred to 2.3 M sucrose in 0.1 M sodium phosphate buffer (pH 7.2) containing 1 % paraformaldehyde for 20 h at 4 °C. For ultracryosectioning, the samples were mounted on top of a metal pin and frozen in liquid nitrogen. Ultracryosectioning and immunogold labelling using an antibody against human alk-SMase (1:2000) were performed as described previously [23]. Controls were performed by omission of the primary antibody.

### Assay of NPP activity and proteins

The activity of NPP was determined as described by Clair et al. [24] and Gijsbers et al. [25] using pNTMP (*p*-nitrophenyl thymidine 5′-monophosphate) and pNPP (*p*-nitrophenyl phenyl phosphate) as substrates in different buffers containing various concentrations of $Ca^{2+}$, $Mg^{2+}$ or $Zn^{2+}$, as described previously [17]. The protein was analysed by a kit from Bio-Rad using albumin as a standard.

### Statistical analysis

Results are expressed as the means $\pm$ S.E.M. for three independent experiments. The statistical significance was analysed by Student's $t$ test and $P < 0.05$ was considered as statistically significant.

## RESULTS

N-glycosylation is critical for alk-SMase activity. Alk-SMase was expressed in COS-7 cells in the presence or absence of tunicamycin, an inhibitor of N-glycosylation. Tunicamycin completely abolished the activity in the expressed enzyme (Figure 2, upper panel) without affecting the expression efficiency (lower panel). Whereas the wild-type alk-SMase shows three bands, the deglycosylated enzyme is a single sharp band. The size difference could be ascribed to the carbohydrate moiety of the wild-type enzyme.

Alk-SMase has five potential N-glycosylation sites and the importance of each site for enzyme activity was examined by site-directed mutation. As shown in Figure 3 (top panel), mutation of each site decreased the alk-SMase activity to various degrees. The most pronounced decrease was seen in the N146 mutation, which almost abolished the enzyme activity, followed by the mutations for N267, N168, N100 and, finally, N121. Western blotting showed that the wild-type enzyme contained mainly three bands, and the band having highest molecular mass might represent the fully glycosylated form (Figure 3, second panel, indicated by an arrow), since the mutated enzymes were mainly lacking this band. The effects of the combined mutations of the glycosylation sites on the activity of alk-SMase are shown in Figure 3 (third panel). Although mutation of N121 alone only decreased the enzyme activity by 25 %, a combination of N100 and N121 decreased the activity by approx. 95 %. Other mutations greater than triple site all abolished the enzyme activity. Western blotting (Figure 3, bottom panel) showed that the molecular mass

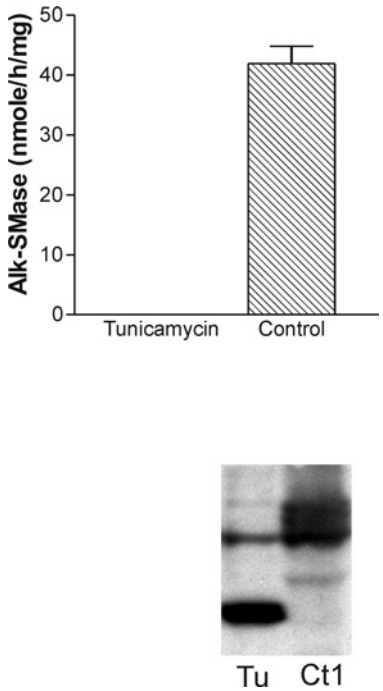


**Figure 2 Effect of tunicamycin on the activity of alk-SMase expressed in COS-7 cells**

COS-7 cells were transfected with wild-type alk-SMase cDNA. Tunicamycin (Tu) was added to the medium 6 h after transfection, to a final concentration of 10 $\mu$g/ml, followed by incubation for 42 h. The control (Ctl) cells were cultured in the absence of tunicamycin. Alk-SMase activities in cell lysates from three separate experiments were determined (upper panel) and the results are presented as means $\pm$ S.E.M. The expressed protein was examined by Western blotting (lower panel).

decreased with increasing degree of deglycosylation. The band with five site mutations is equal to that expressed in COS-7 cells in the presence of tunicamycin.

### Deglycosylation affects the transport of alk-SMase in cells

The glycosylation was found to affect not only the activity but also the transport of the enzyme to the plasma membrane. As shown in Figure 4 (top panel), the activities of the expressed alk-SMase with the C-terminal anchor truncated (R440) or the exon 5-encoded domain truncated (T415) were significantly increased in the cell culture medium when compared with the wild-type enzyme. Furthermore, Western blotting showed the appearance of the truncated enzymes in the medium (middle panel). However, when the five glycosylation sites in the C-terminal anchor-truncated enzyme were further mutated, no alk-SMase activity could be found in the medium and no alk-SMase protein could be identified in the culture medium by Western blotting (bottom panel). Similar effects were also obtained when the C-terminal-truncated enzyme was expressed in COS-7 cells in the presence of tunicamycin (results not shown).

Immunogold labelling was performed to detect the cellular localization of alk-SMase after deglycosylation. In transfected COS-7 cells, the wild-type alk-SMase was found in high concentration at the plasma membrane (Figure 5A). The wild-type enzyme was also found at a low concentration in endosome-like structures (Figure 5B). As seen in Figure 5(C), the C-terminal-truncated alk-SMase was not found in the plasma membrane. Meanwhile, we found an increase of the enzyme in vesicular structures, which probably represents transport vehicle en route to the

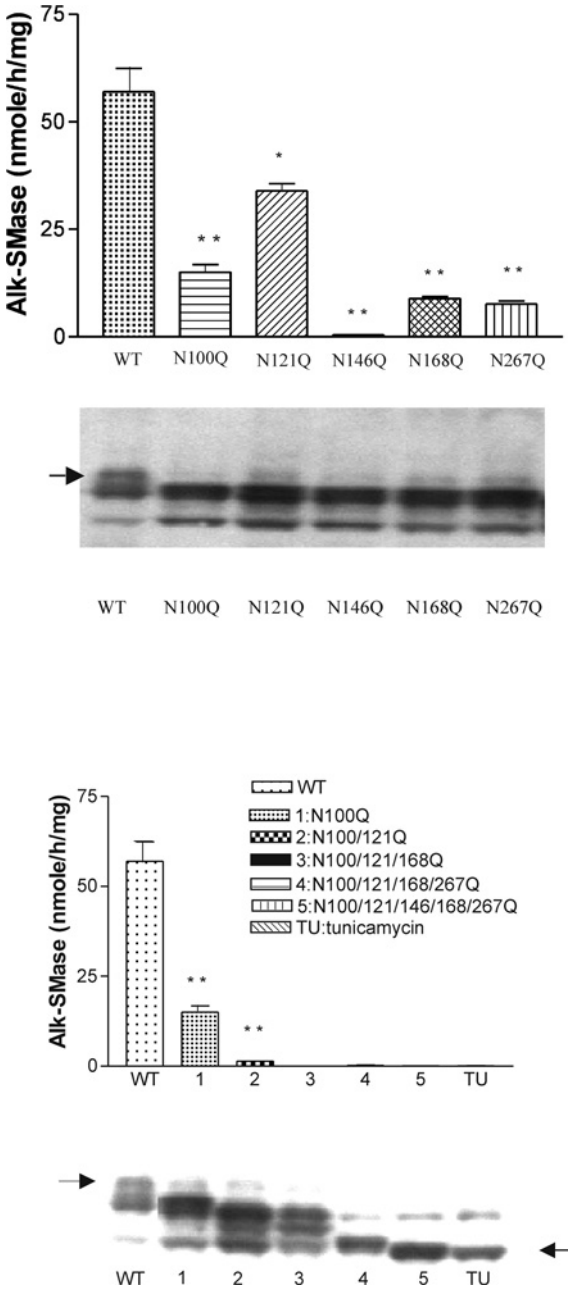


**Figure 3 Alk-SMase activity after mutation of the potential N-glycosylation sites**

The potential glycosylation sites were mutated individually and the enzyme was expressed in COS-7 cells. The changes in alk-SMase activity in cell lysate were measured (top panel) and the proteins expressed were examined by Western blotting (second panel). The predicted fully glycosylated alk-SMase form is indicated by an arrow. In the third panel, the mutation was performed in an additive manner as indicated in the Figure. The alk-SMase activities in the expressed enzyme were determined. The changes in the molecular mass were examined by Western blotting. The predicted fully glycosylated and deglycosylated forms are indicated by an arrow on the left and right respectively (bottom panel). Results are expressed as the means $\pm$ S.E.M. for three independent experiments. $^{**}P < 0.01$ or less. The statistical significance was not analysed for samples 3, 4, 5 and tunicamycin (TU) in the bottom panel, owing to absence of the activity.

plasma membrane (Figure 5C) or to the endosomes (Figure 5D). In COS-7 cells transfected with the C-terminal-truncated alk-SMase with five glycosylation sites mutated, the enzyme was seldom present in the plasma membrane (Figure 5E), but was in high

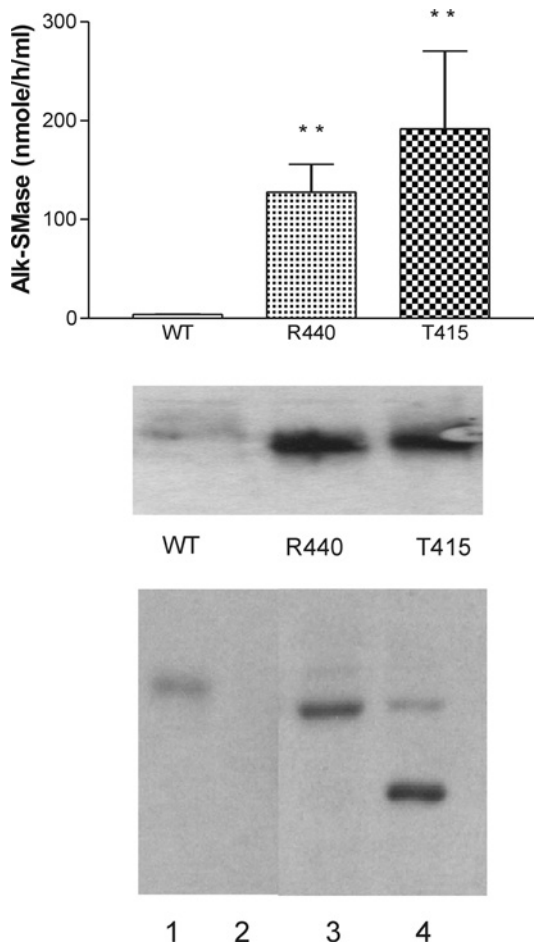


**Figure 4 Release of C-terminal-truncated alk-SMase into the culture medium**

Wild-type (WT) alk-SMase or C-terminal-truncated enzyme was expressed in COS-7 cells. After 48 h, the enzyme activity in the culture medium was determined (top panel). R440, the C-terminal anchor from P441 to A458 was truncated; T415, the C-terminal from E416 to A458 encoded by exon 4 was truncated. Results are expressed as the means ± S.E.M. for three independent experiments. $^{**}P < 0.01$ compared with WT. No statistical significance was identified between R440 and T415. In the middle panel, 10 $\mu$l of the medium from each group was subjected to Western blotting. The bottom panel shows the effect of deglycosylation on the release of alk-SMase into the medium. Both the C-terminal anchor-truncated alk-SMase and the enzyme with glycosylation sites further mutated were expressed in COS-7 cells. Either 10 $\mu$l of the medium or 50 $\mu$g of the cell lysate was subjected to Western blotting. Lane 1, medium, C-terminal-truncated; lane 2, medium, C-terminal-truncated, with mutant glycosylation sites; lane 3, lysate of COS-7 cells expressing C-terminal-truncated alk-SMase; lane 4, lysate of COS-7 cells expressing C-terminal-truncated enzyme with mutant glycosylation sites.

concentration in the endosomes (Figure 5F), probably reflecting degradation of the protein.

### Effects of introducing the NPP core in alk-SMase

Alk-SMase shares 32 % similarity with human NPPs but has no detectable NPP activity [17]. The sequence TKTFPNH in the predicted active core of NPP has been changed to TMTSPCH in alk-SMase. We performed site-directed mutations to restore the NPP core sequence in alk-SMase individually and totally, followed by the determination of alk-SMase and/or NPP activities. As shown in Figure 6, substitution of M74K, S76F and C78N in this part of the enzyme reduced the alk-SMase activity, with the mutation of S76F abolishing the activity totally. T75 is predicted to be a key residue in NPPs and mutation of this residue also leads

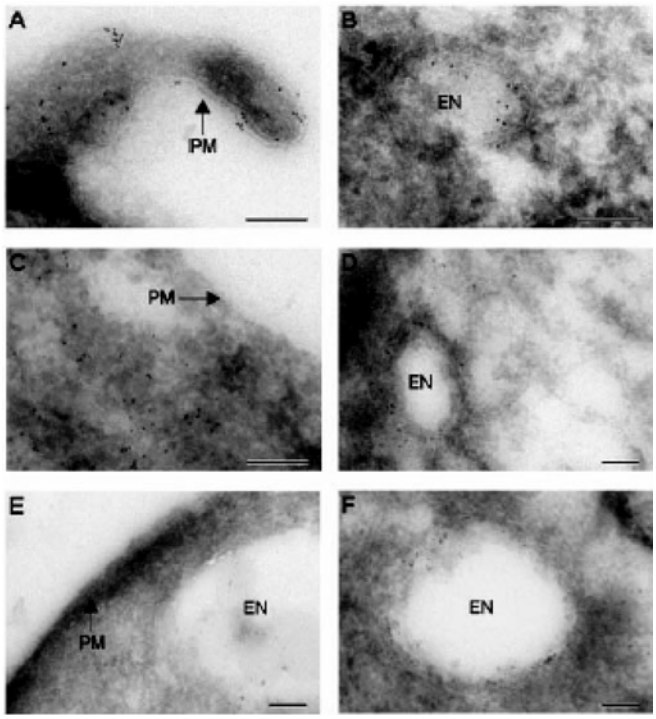


**Figure 5 Immunogold labelling of alk-SMase in COS-7 cells transfected with wild-type alk-SMase (A, B), C-terminal-truncated alk-SMase (C, D) and C-terminal-truncated alk-SMase with five glycosylation sites mutated (E, F)**

(**A**, **B**) The enzyme is mainly found in the plasma membrane (PM) and only in low concentration in endosome (EN). (**C**, **D**) The truncated enzyme is absent from the PM and found in vesicular structures (**C**) and EN. (**E**, **F**) The truncated alk-SMase with deglycosylation is absent from the plasma membrane and found in high concentration in the endosomes. Scale bars, 200 nm.

to loss of SMase activity. However, none of the mutated forms of the enzyme showed activity against pNTMP (results not shown). Total substitution of the activity core sequence TMTSPCH of alk-SMase with the one of NPPs, i.e. TKTFPNH, also did not lead to the appearance of NPP activity in alk-SMase. As shown in Table 2, COS-7 cells have endogenous NPP activity when measured in the presence of $Mg^{2+}$, $Ca^{2+}$ or $Zn^{2+}$. Transfection of the alk-SMase having an activity core identical with NPP did not induce any increase in NPP activity.

### Critical effects of the putative metal-binding sites on alk-SMase activity

Alk-SMase has six conserved amino acid residues that form two metal co-ordinating sites in NPPs [25]. Since alk-SMase is not dependent on metal ions, the importance of these sites is unknown. We destroyed the binding sites by mutation of one residue in each triad and found that the mutation abolished alk-SMase activity (Figure 7). We then measured the SMase activity or NPP activity of the mutated enzyme in the presence of $Ca^{2+}$, $Mg^{2+}$ or $Zn^{2+}$ up to 2 mM; no recovery of the enzyme activities was identified (results not shown).

## DISCUSSION

Intestinal alk-SMase hydrolyses SM in the gut and generates multiple lipid messengers that probably affect intestinal functions. Previous studies have shown that the enzyme has antiproliferative effects [26] and the activity of alk-SMase is decreased in sporadic colon adenomas, colorectal carcinomas, familial adenomatous polyposis and longstanding ulcerative colitis [8–10]. It is therefore

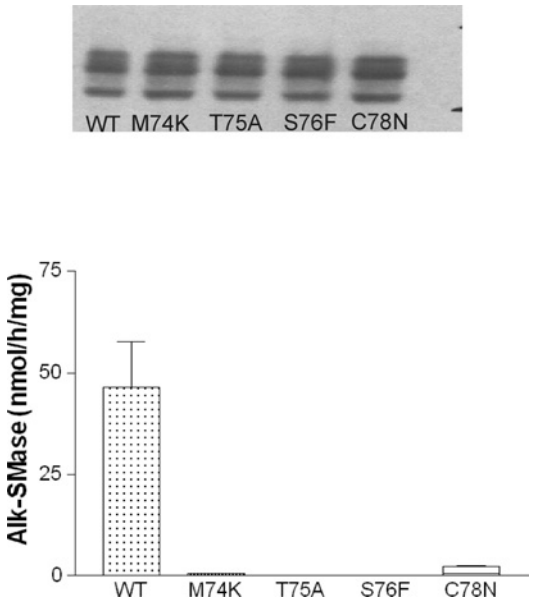


**Figure 6 Changes of alk-SMase activity by mutation of the predicted activity core**

The amino acid residues in the predicted active core were mutated individually to the corresponding ones in most NPPs. The expressed proteins were examined by Western blotting (upper panel) and alk-SMase activity in the cell lysate was assayed (lower panel). Results were obtained from three independent experiments and are presented as means ± S.E.M.

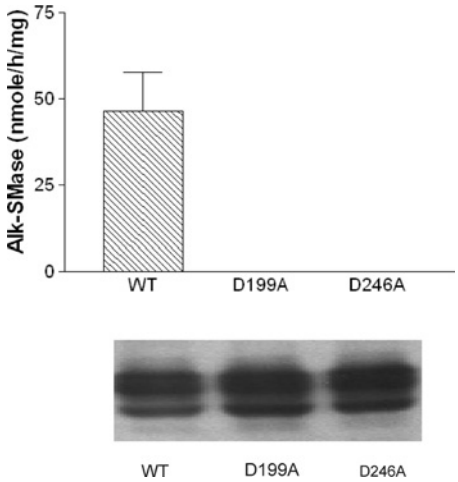


**Figure 7 Effects of mutation of the putative metal-binding sites on alk-SMase activity**

COS-7 cells were transfected with either wild-type alk-SMase or alk-SMase in which one of the residues forming the two triad metal-binding sites was mutated. Alk-SMase activity in the cell lysate was determined (upper panel) and the expressed proteins were examined by Western blotting (lower panel). Results were obtained from three separate experiments and are presented as means ± S.E.M.

important to characterize how the enzyme acts and how it is regulated. The present study was undertaken to examine the functional roles of glycosylation, and the putative metal-binding site, the predictive active core and the C-terminal domain in alk-SMase function.

Human alk-SMase has five potential glycosylation sites [17]. Using ECL® advanced reagent, Western blotting of wild-type alk-SMase often shows 2–3 bands, indicating that glycosylation in the wild-type enzyme is heterogeneous. The band with the highest molecular mass may represent the fully glycosylated form, since it disappeared when any of the five glycosylation sites was mutated. The stepwise reduction of the molecular mass with increase in mutation number of potential glycosylation sites indicates that all five sites are glycosylated.

The present study shows that glycosylation of alk-SMase is critical for the enzyme activity because the expressed alk-SMase in COS-7 cells had no activity when the host cells were treated with tunicamycin, an inhibitor of N-glycosylation. The decrease in molecular mass caused by tunicamycin is equivalent to the carbohydrate moiety of the enzyme. Individual mutations of the N-glycosylation sites showed that the most important glycosylation site is N146 and the least is N121. However, mutations combining N121 with any other one or two glycosylation sites almost abolished the enzyme activity.

We recently showed that truncation of the C-terminal anchor of alk-SMase significantly increased the enzyme activity in cell culture medium when expressed in COS-7 cells [18], indicating that the enzyme is transported to the plasma membrane and hooked on the membrane through its C-terminal. This was confirmed by our immunochemical studies, which showed a high concentration of the wild-type alk-SMase in the plasma membrane and the disappearance of the enzyme when the C-terminal anchor was truncated. The present study also showed an increased concentration of the C-terminal-truncated alk-SMase in intracellular vesicles. Because the previous study [18] did show a large increase in the activity of the truncated enzyme in cell culture medium, the present finding of an increased amount of enzyme in vesicles may reflect the increase in synthesis and transport of the enzyme to the plasma membrane when the amount of enzyme in this compartment is decreased.

The release of the C-terminal-truncated alk-SMase into the medium was abolished by mutation of the glycosylation sites or by tunicamycin. The deglycosylated alk-SMase was almost

**Table 2 NPP activity (absorbance · $h^{-1}$ · $mg^{-1}$) of wild-type alk-SMase and alk-SMase with the active core mutated**

COS 7 cells were transfected with wild-type alk-SMase and alk-SMase with whose active core TMTSPCH has been substituted with TKTFPNH, the one conserved in NPPs. A cell-free extract was prepared and NPP activity was determined using both pNTMP and pNPP as the substrate under different assay conditions. Results were obtained from three independent experiments and are expressed as means ± S.E.M. No statistical significance was identified in each compared group.

| | pNTMP | | pNPP | |
|---|---|---|---|---|
| Assay conditions | Control | Mutant | Control | Mutant |
| 50 mM Tris/HCl, 0.15 M NaCl, 2 mM EDTA and 10 mM TC, pH 9.0 | 0.46 ± 0.16 | 0.36 ± 0.17 | 0.82 ± 0.20 | 0.62 ± 0.19 |
| 50 mM Tris/HCl, 0.15 M NaCl, 1 mM $Ca^{2+}$ and 1 mM $Mg^{2+}$, pH 9.0 | 6.55 ± 0.12 | 5.84 ± 0.46 | 1.53 ± 0.13 | 1.42 ± 0.04 |
| 50 mM Tris/HCl, 0.2 M KCl and 1 mM $Ca^{2+}$, pH 9.0 | 2.17 ± 0.09 | 2.61 ± 0.39 | 2.98 ± 0.16 | 2.92 ± 0.29 |
| 50 mM Tris/HCl, 0.15 M NaCl and 2 mM $Zn^{2+}$, pH 9.0 | 9.95 ± 0.40 | 10.53 ± 1.07 | 1.52 ± 0.11 | 1.48 ± 0.13 |

exclusively located in endosomes. The results indicate that N-glycosylation also affects the transport of the enzyme to the plasma membrane and abnormal glycosylation of the enzyme results in an accumulation of the enzyme in endosome-like structures probably for degradation.

Apart from glycosylation, the function of alk-SMase is affected by the residues forming metal-binding sites in NPP. It is not known why alk-SMase conserved these residues forming the metal-binding sites, since previous studies did not find any effect of $Ca^{2+}$ and $Mg^{2+}$ or EDTA and EGTA on alk-SMase activity [14,15,27]. Although the possibility that metal ions have already been tightly bound to the sites and are hard to remove cannot be excluded, adding the metal ions back to the binding site mutant enzyme did not recover the enzyme activity. The results differed from the study on NPPs [28], indicating that these binding sites in alk-SMase may have other functions. We previously speculated that these residues may form a binding pocket for positively charged SM [17]. Gijsbers et al. [25] proposed that the metal-binding site in NPP may also be important in stabilizing the NPP1 enzyme. We recently identified an exon deletion of alk-SMase in HT29 cells, leading to inactivation of alk-SMase. The deleted exon contains the codon for $His^{353}$ that is another residue that participates in the formation of the metal-binding sites. Site mutation of $His^{353}$ also completely abolished the enzyme activity [29].

Although alk-SMase shares approx. 32 % identity with human NPPs, the enzyme so far has not been found to exhibit any activity against the substrates of NPPs under various assay conditions. The active core of NPP was predicted to be TKTFPNH (residues 254–260) and the $Thr^{256}$ is considered to be critical. In alk-SMase, the corresponding core is TMTSPCH (residues 73–79), in which three residues have been modified and the critical Thr (T75) conserved. The present study showed that the modified domain is crucial for alk-SMase activity, as any single site mutation of these residues sharply decreased alk-SMase activity, with T75 and S76 being most critical. However, the substrate specificity between alk-SMase and NPP does not depend on the structure of the active core only, because substitution of the sequence in alk-SMase with that in NPP did not give the enzyme NPP activity. Recently, Cimpean et al. [28] have studied the substrate specificity of NPP1 and NPP2 by mutagenesis. Npp1 is an active NPP member and NPP2, differing from NPP1, has activity against lysophosphatidylcholine and sphingosine-1-phosphate [30]. In agreement with our findings with alk-SMase, the substrate specificity between NPP1 and NPP2 is not simply dependent on a few residues but probably determined by the whole polypeptide chain.

Alk-SMase has six exons and five of them are translated. The minimum functional domain of alk-SMase is currently unknown. The present study shows that truncation of the C-terminal anchor (17 residues) or the C-terminal domain (43 residues) encoded by exon 5 did not cause a significant lowering of the enzyme activity. However, the domain encoded by exon 4 is critical, since skipping the transcription of exon 4 in human colon cancer HT29 cells resulted in inactivation of alk-SMase [29].

Alk-SMase has been considered as a stable enzyme [27,31], which can even be purified from the intestinal content collected from ileostomy stomas [16]. However, a decrease in the enzyme activity has been reported for several diseases related to colonic tumorigenesis [8–10]. In addition to the mis-transcription reported recently in HT29 cells [29], the present study shows that the normal function of alk-SMase relies on a rigid structure, which is maintained by the carbohydrate moiety, the putative metal-binding sites and the predicted activity core. Glycosylation is known to be closely related to carcinogenesis and abnormal glycosylation has been frequently found in inflammatory bowel diseases [32,33], which is linked to an increased risk of colon cancer. Our study thus provides not only novel information on function–structure relationships of alk-SMase, but also sheds light on the decrease in enzyme activity associated with colon cancer.

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