Identification of Human Intestinal Alkaline Sphingomyelinase as a Novel Ecto-enzyme Related to the Nucleotide Phosphodiesterase Family

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Alkaline sphingomyelinase (alk-SMase) hydrolyzes dietary sphingomyelin and generates sphingolipid messengers in the gut. In the present study, we purified the enzyme, identified a part of the amino acid sequence, and found a cDNA in the GenBank™ coding for the protein. The cDNA contains 1841 bp, and the open reading frame encodes 458 amino acids. Transient expression of the cDNA linked to a Myc tag in COS-7 cells increased alk-SMase activity in the cell extract by 689-fold and in the medium by 27-fold. High activity was also identified in the anti-Myc immunoprecipitated proteins and the proteins cross-reacted with anti-human alk-SMase. Northern blotting of human intestinal tissues found high levels of alk-SMase mRNA in the intestine and liver. The amino acid sequence shared no similarity with acid and neutral SMases but was related to the ecto-nucleotide phosphodiesterase (NPP) family with 30–36% identity to human NPPs. Alk-SMase has a predicted signal peptide domain at the N terminus and a signal anchor domain at the C terminus. The ion-binding sites and the catalytic residue of NPPs were conserved, but the substrate specificity domain was modified. Alk-SMase had no detectable nucleotidase activity, but its activity against sphingomyelin could be inhibited by orthovanadate, imidazole, and ATP. In contrast to NPPs, alk-SMase activity was not stimulated by divalent metal ions but inhibited by Zn2+. Differing from NPP2, the alk-SMase cleaved phosphocholine but not choline from lysophosphatidylcholine. Phylogenetic tree indicated that the enzyme is a new branch derived from the NPP family. Two cDNA sequences of mouse and rat that shared 83% identity to human alk-SMase were identified in the GenBank™. In conclusion, we identified the amino acid and cDNA sequences of human intestinal alk-SMase, and found that it is a novel ecto-enzyme related to the NPP family with specific features essential for its SMase activity.

Sphingomyelin (SM)³ is a component of all mammalian cell membranes particularly the plasma membrane and the lysosomal membrane. SM is also a dietary component and is mainly present in milk, eggs, meat, and marine products (1, 2). Hydrolysis of SM generates ceramide, sphingosine, and sphingosine 1-phosphate that have regulatory effects on numerous cellular functions such as proliferation, differentiation, and apoptosis (3, 4). At least five types of sphingomyelinase (SMase) have been identified, of which acid and neutral SMases have been cloned (5–9). An enzyme that catalyzes hydrolysis of SM with optimal alkaline pH was first identified in the intestinal content of human and intestinal mucosa of rat and pig by Nilsson (10) and was named alkaline SMase (alk-SMase) thereafter (11). Previous studies indicated that alk-SMase may be responsible for digestion of dietary SM and for hydrolysis of endogenous SM derived from bile and from the brush borders of sloughed mucosal cells.

SM metabolism in the intestine may have implications in colon cancer development. Dietary supplement with SM and ceramide analogues was found to inhibit development of chemically induced colon cancer in animal studies (12–14). We previously found that alk-SMase activity was significantly reduced in the tissues of human colon adenomas and carcinomas (15, 16) and in the mucosa of longstanding ulcerative colitis (17), indicating that the reduced SM hydrolysis in the gut may increase the susceptibility of the colonic mucosa to carcinogenic factors. SM metabolism in the gut may also affect cholesterol absorption, because both dietary SM in the gut (18, 19) and hydrolysis of SM in the membrane of enterocytes by SMase (20) were found to inhibit cholesterol absorption.

We recently purified and characterized alk-SMase from rat small intestine (21) and human ileostomy content (22). The enzyme has several properties that distinguish it from acid and neutral SMases. Its activity was not dependent on divalent ions such as Mg²⁺ and Ca²⁺, not inhibited by glutathione, and was resistant to trypsin digestion (11, 21). The enzyme activity was specifically dependent on taurocholate and taurochenodeoxycholate, two taurine-conjugated primary bile salts, and was inhibited by other common detergents such as Triton X-100.

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The abbreviations used are: SM, sphingomyelin; alk-SMase, alkaline sphingomyelinase; CHAPS, 3-[3-cholamidopropyl]dimethylammoniomol-1-propanesulfonic acid; TC, taurocholate; SMase, sphingomyelinase; NPP, nucleotide phosphodiesterase; pNPP, p-nitrophenyl phosphophosphate; pNTMP, p-nitrophenyl thymidine 5′-monophosphate; MALDI, matrix-assisted laser desorption ionization; PMSF, phenylmethylsulfonyl fluoride; PC, phosphatidylcholine.

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and CHAPS (21). By using the immunologol technique, the enzyme was demonstrated on the surface of human intestinal brush borders, in endosome-like structures, and in Golgi complexes (22). Western blotting of rat tissues only found the enzyme protein in the intestine and not in other organs including brain, heart, lung, liver, kidney, spleen, and pancreas (21). Western blot also demonstrated that the enzyme protein was reduced in human colon cancer tissues compared with the surrounding normal tissues (22).

In that way, from the purified enzyme we identified the full amino acid and cDNA sequences for the human intestinal alk-SMase. We found that the enzyme is a novel eukaryote enzyme related to the nucleotide phosphodiesterase (NPP) family with specific features in terms of substrate specificity and metal ion dependence.

**MATERIALS AND METHODS**

Human small intestinal contents were collected from ileostomy stomas of six individuals as described (22). Bovine milk sm was provided by Dr. Lena Nyberg at Skåne Dairy Co. (Malmö, Sweden) and labeled with [14C]-CH$_3$ as described (23). DEAE-Sepharose, phenyl-Sepharose 6 FF, [32P]dCTP, 1HCholine chloride, and protein G-Sepharose were purchased from Amersham Biosciences. Uno Q anion exchange chromatography cartridge, pre-packed SE gel chromatography column, biologic HR protein purification system, and isoelectrofocusing instrument Rotofor were developed in AgriSera AB (Vännäs, Sweden) using our purified human alk-SMase (22). Human digestive system Northern blot was purchased from Clontech (Palo Alto, CA). p-Nitrophenyl thymidine 5'-monophosphate (pNTPM), p-nitrophenyl phenylphosphate (pNPP), taurouctone (TCT), ATP, ADP, AMP, neutral SMase from Bacillus cereus, and other chemicals used in characterization were purchased from Sigma.

**SMase Assay—**Alk-SMase activity was determined by methods described previously (24). In the purification experiment, 5 µl of samples were mixed with 95 µl of 50 mM Tris-HCl buffer, pH 9.0, containing 0.15 M NaCl, 2 mM EDTA, 10 mM TC (assay buffer), 0.1 mM SM, and 0.80 µM [14C]SM (~8000 dpm) and incubated at 37°C for 30 min. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2:1) followed by centrifugation at 10,000 rpm for 10 s. An aliquot (100 µl) was extracted by adding 0.4 ml of chloroform/methanol (2:1) followed by Voyager DE Pro instrument (Applied Biosystems). Identification was based on comparison of theoretical mass values for tryptic fragments derived from the cDNA sequence and the masses detected in the in-gel digest (26).

**Transient Expression—**Based on the information of amino acid sequence (415 amino acid residues) obtained from the purified enzyme, we found that in the GenBank database an incomplete cDNA segment (clone ID, IMAGE 5186743) which was derived from a pool of colon, kidney, and spleen cDNA was encoded our alk-SMase sequence. Amplification of the DNA clone (pCMV-Sport 6) was then found commercially available and was purchased from ResGen. The cDNA insert of the pCMV-Sport 6 was amplified and sequenced. The translated amino acid sequence of the cDNA was compared with that obtained from purified human alk-SMase. To confirm that the cDNA does encode for human alk-SMase, COS-7 cells were incubated in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal calf serum and 2 mM glutamine. The cDNA insert in pCMV-Sport 6 was amplified by PCR using primers 5’-TCG-GTACCGGAAAGGATGAGGGGCGCGTCAGCTC-3’ and 5’-TACGG-GCCGCCCTCGACGACGACGAGAAAT-3’ and was cloned into the mammalian expression vector pCNA4/TO/myc-His B at BamHI and NotI sites. The constructed plasmid was co-transfected with the control plasmid in the presence of LipofectAMINE, followed by incubation of the cells for 48 h. Control cells were transfected with the mock plasmid in the same way as the transfected cells. At the end of incubation, the cell culture medium was collected, and the cells were lysed by a 50 mM Tris-HCl buffer containing 1 mM PMSF, 2 mM EDTA, 0.5 mM dithiothreitol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, and 10 mM TC, followed by sonication for 10 s. After centrifugation at 12,000 relative centrifugal force for 10 min at 4°C, the alk-SMase activity in the cell-free extract as well as in the cell culture medium of both transfected and control cells was determined. To confirm pH optimum, the SMase activities in the lysate of transfected cells were assayed in buffers with various pH values as described previously (24).

**Immunoprecipitation and Western Blot—**The lysate of COS-7 cells (100 µl) after transfection was first pre-cleared with 20 µl of protein G-Sepharose at 4°C for 1 h. After centrifugation, the supernatant was incubated with 1 µl of anti-Myc antibody for 1 h and then with 20 µl of protein G-Sepharose beads (50% suspension) for 2 h. The Sepharose beads were precipitated by centrifugation and washed 3 times with 1 ml of lysis buffer. The Sepharose beads were then resuspended in 10 µl of lysis buffer, and alk-SMase activity in 5 µl of the suspension was determined. Another aliquot of the Sepharose beads (10 µl) was boiled in electrophoresis sample buffer, subjected to 10% SDS-PAGE, and then transferred to nitrocellulose membrane by electrophoresis. The membranes were incubated with anti-Myc antibodies. The cDNA clone (pCMV-Sport 6) was found commercially available from Bacillus cereus.

**Determination of SM, Phosphatidylcholine (PC), and Lyso Phosphatidylcholine (Lyso-PC) in Cells—**The determination of SM, PC, and lyso-PC in cells was performed as described previously (27). In brief, COS-7 cells were labeled with [1H]Choline chloride (0.5 µCi/ml) for 48 h and then transfected with the cDNA of alk-SMase or the vector alone as control. After 48 h of incubation, the total lipids in the cells were extracted and applied on a Silica Gel plate (60F, 0.25 mm) for thin layer chromatography. The plates developed by 25 mM ammonium hydroxide (65:25:4, v/v/v) and the SM, PC, and lyso-PC bands were visualized by iodine vapor. The bands were scraped according to the internal standard, and the radioactivities in the bands were determined by liquid scintillation.

**Phylogenetic Tree—**The protein sequences of several members of the SMase family and related enzymes were aligned using the ClustalW software (Applied Biosystems). Edman degradation of polyvinylidene fluoride-bound peptides was carried out with a Procise CLC sequencer (Applied Biosystems) (26). MALDI mass spectrometry of tryptic fragments from in-gel digestion was carried out using Voyager DE Pro instrument (Applied Biosystems).
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Fig. 1. Purity of human intestinal alk-SMase after purification. Lane A, standard protein. Lane B, the original material for purification. Lane C, the purified human intestinal alk-SMase. Lane D, the purified rat intestinal alk-SMase. The proteins were resolved by 10% SDS-PAGE, and the gel was stained by silver staining.

RESULTS

Purification of Human Alk-SMase and Amino Acid Sequence—The procedure for alk-SMase purification followed the methods described recently, and the results obtained were similar (21, 22). In brief, the enzyme was not retained in DEAE-Sepharose column equilibrated with 20 mM Tris-HCl buffer containing 0.15 M NaCl and 1 mM Mg2+ containing 0.15 M NaCl, 1 mM Ca2+ and 1 mM Mg2+ (30, 31). In a third experiment, activity was assayed in 50 mM Tris buffer, pH 8.0, containing 0.2 M nitritrophic acid and imidazole were studied by assaying the enzyme activity in the assay buffer with the presence of the substances. Whether alk-SMase has NPP activities was examined by using pNTP and pNPP as substrates in four different buffers with various dilution ions as described below. First the NPP activity of alk-SMase was determined in the buffer optimal for its activity against SM. In a second experiment, the activity was assayed in 50 mM Tris-HCl buffer, pH 9.0, containing 0.15 M NaCl, 1 mM Ca2+, and 1 mM Mg2+ (30, 31). In a third experiment, activity was assayed in 50 mM Tris buffer, pH 8.0, containing 0.2 M KCl and 1 mM Ca2+ (32). Finally, the activities were assayed in 50 mM Tris buffer containing 0.15 M NaCl with different concentrations of Zn2+ ranging from 0.0125 to 2 mM. In all determinations described above, 1 mM pNTP or pNPP was present as substrate, and the production of p-nitritrophic acid was kinetically analyzed at 405 nm every 15 min up to 1 h on a microplate reader (Bio-Rad).

Activity of Alk-SMase against Lyso-PC—To examine whether alk-SMase has activity against lyso-PC, purified alk-SMase (30 ng) was incubated with 25 nmol of [14C]lyso-PC in 50 mM Tris buffer, pH 7.5, containing 6 mM TC, 1 mM Mg2+, 1 mM Ca2+ to a final volume of 200 μl at 37 °C for 1 h. The reaction was terminated by adding 0.8 ml of chloroform/methanol (2:1). The upper phase was acidified by acetic acid to pH about 4 and discarded after stirring and centrifugation. The lower phase was washed with chloroform/methanol (1:1), and applied on a Silica gel plate (60F, 0.25 mm) for thin layer chromatography. The plates were developed with chloroform/methanol/acetic acid/H2O (50:40:3:0.6) and stained with iodine vapor. The bands of lyso-PC and lyso-PA were scraped according to the positions of standards lyso-PC and lyso-PA. Under these conditions, the monoglyceride migrates with the solvent front and was also scraped. The radioactivity in each band was determined by liquid scintillation.

The changes of SM, PC, and lyso-PC after the transfection in COS-7 cells prelabeled with [3H]choline chloride were determined in three separate experiments. The transfection decreased labeled SM by 14.1 ± 0.5% (p < 0.0001), labeled lyso-PC by 12.2 ± 3.5% (p < 0.05), and labeled PC by 7.9 ± 1.1% (p < 0.05) as compared with the control cells.

Full cDNA and Amino Acid Sequences of Alk-SMase—The full sequence of the cDNA insert of pCMV-Sport 6 was determined. It contained 1377-bp coding sequence and 20 nucleotides of 5′-untranslated region and 444 nucleotides of 3′-untranslated region except for the poly(A) sequence. The coding sequence is identical to that of IMAGE 5186743, and the full-length sequence has been now registered as human intestinal alk-SMase in GenBank™ (AY230663). The gene was found to be located in chromosome 17 with 6 exons. The open reading frame of the cDNA codes 458 amino acid residues that are shown in Fig. 5, and the 415 amino acids from the N terminus are identical to the sequence we obtained from the purified enzymed by MALDI mass mapping and Edman degradation. By this method, we were able to obtain a sequence that consisted of 415 amino acid residues as shown in Fig. 2.

Transient Expression—NCBI searching with the amino acid sequence obtained, we found a plasmid (pCMV-Sport 6) from ResGen containing a cDNA insert that encodes our sequence. We amplified the cDNA insert, cloned it into an expression vector with a Myc tag, and transfected COS-7 cells. Both the cell number and cell protein concentration did not change significantly 48 h after transfection. However, as shown in the top panel of Fig. 3, the alk-SMase activity was markedly increased in the cDNA-transfected cells by about 689-fold. The activity was also increased by 27-fold in the culture medium of transfected cells (middle panel). When the activity of the cell-free extract was determined in the buffers with different pH values, similar to purified alk-SMase (21, 22), the expressed enzyme showed the highest activity at pH 9.0 (bottom panel).

To confirm whether the expressed protein did have SMase activity, the protein was precipitated by the antibody against the Myc tag, and the alk-SMase activity was determined in the immunoprecipitated proteins. As shown in the left panel of Fig. 4, high alk-SMase activity was identified in the precipitated proteins in the cDNA-transfected cells. On the right panel, the proteins were further subjected to Western blot. Anti-Myc antibody identified a major protein of about 60 kDa in the cDNA-transfected cells (lane C), and the protein strongly cross-reacted with anti-human alk-SMase antibody (lane D).

The changes of SM, PC, and lyso-PC after the transfection in COS-7 cells prelabeled with [3H]choline chloride were determined in three separate experiments. The transfection decreased labeled SM by 14.1 ± 0.5% (p < 0.0001), labeled lyso-PC by 12.2 ± 3.5% (p < 0.05), and labeled PC by 7.9 ± 1.1% (p < 0.05) as compared with the control cells.
human alk-SMase (Fig. 2). Searching against PROSITE (33) demonstrated that the enzyme has five potential glycosylation sites (residues 100, 121, 146, 168, and 267), seven protein kinase C phosphorylation sites (residues 84, 102, 157, 236, 269, 372, and 438), four casein kinase II phosphorylation sites (residues 157, 184, 250, and 269), and six N-myristoylation sites (residues 92, 134, 144, 151, 161, 446). The protein has two Cys (Cys-78 and Cys-392) located at N and C termini, respectively. The transmembrane segments were predicted by TMpred. In both N and C termini, there is a highly hydrophobic transmembrane domain (residues 5–22 and 441–457, respectively). By use of SignalP V.20 (34), the N-terminal hydrophobic domain is a predicted signal peptide with a cleavage site between residue 21 and 22 and that of C-terminal is a predicted Signal anchor.

Relation to NPP Family
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BLAST searching in GenBank™ found that the amino acid sequence of alk-SMase exhibited 30–36% identities to NPPs but no similarity with either neutral or acid SMases. Fig. 5 also shows the multiple alignment of human alk-SMase to human NPPs. The catalytic residue threonine in the active site of NPPs is conserved in human alk-SMase (Thr-75). The amino acid sequence of the motif (TKTF-PNH) adjacent to Thr-75 of NPPs has been modified in alk-SMase, with Lys replaced by Met, Phe replaced by Ser, and Asn by Cys. According to the three-dimensional structure model for NPP1 predicted by Gijsbers et al. (31), there are two metal-coordinating sites formed by Asp-358, His-362, and His-517, and by Asp-405, His-406, and Asp-405, respectively. All these amino acids are conserved in alk-SMase as shown in shaded boxes on the figure.

BLAST searches with amino acid sequence on GenBank™ identified three recently submitted or modified sequences: AAH41453, XP_221184, and XP_137914 with unknown functions. AAH41453 is an updated human protein encoded by the cDNA clone IMAGE 5186743. Its sequence is identical with human alk-SMase. XP_221184 is a predicted rat protein, and XP_137914 is a predicted mouse protein. The alignment of human alk-SMase to the rat and mouse proteins is shown in Fig. 6. The two proteins are 83% identical with human alk-SMase with an identical motif sequence (TMTPC) as human alk-SMase around the catalytic residue threonine. The amino acids that are important for metal-binding sites are also conserved in these proteins. The results indicate that the two proteins are alk-SMase in rat and mouse, respectively.

Evolutionary tree derived from protein sequences in Swiss-Prot is shown in Fig. 7. The bootstrap support values are all above 80%. The rat and mouse proteins are located on the same branch as alk-SMase and not as any of the NPPs. This supports the concept that these two proteins are alk-SMase homologues in rat and mice. The alk-SMase is close to human NPP5 but is still a new branch of the tree.

Expression of Alk-SMase in the Gut
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We showed previously that alk-SMase was only found in the intestine in many species and additionally in human bile (35, 36). Northern blotting of total RNA isolated from human gastrointestinal tissues was performed. The results are shown in Fig. 8. High levels of alk-SMase mRNA were identified in duodenum, jejunum, and ileum. By this method, the level of mRNA was hardly detected in esophagus, stomach, and colon.

Characterization of Human Alk-SMase
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Our previous studies on purified enzyme have shown that the properties of alk-SMase differed considerably from the acid and neutral SMase in many respects (11, 21, 22) as summarized in Table I. Based on the novel finding that the enzyme is related to the NPP family, we examined whether purified human alk-SMase has nucleotidase activity. Under four different conditions using two types of substrates, we failed to identify nucleotidase activity of the purified alk-SMase (Table II). For comparison, rat small
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FIG. 4. The SMase activity and Western blotting of immunoprecipitated proteins. COS-7 cells were transfected with the predicted alk-SMase cDNA in pCDNA4/TO/myc-His. The control cells were transfected with the plasmid alone. After 48 h of incubation, the cell-free extracts were immunoprecipitated by anti-Myc antibody and protein G-Sepharose beads. The alk-SMase activity in 5 μl (50% suspension) of Sepharose beads was determined (left panel). The immunoprecipitated proteins were subjected to Western blot with anti-Myc and anti-human alk-SMase antibodies (right panel). Lane A, prestained standard proteins (Bio-Rad); lane B, sample from control cells blotted with anti-Myc antibody; lane C, sample from transfected cells blotted with anti-Myc antibody; lane D, the same sample as lane C blotted with anti-human alk-SMase antibody.

intestinal mucosa and human colonic biopsies samples were also assayed and found to be able to hydrolyze both pNTMP and pNPP.

A general feature of NPPs is the dependence of activity on divalent ions. The effects of divalent ions on alk-SMase were therefore studied. We found that the alk-SMase activity was significantly inhibited by Zn2+, and the inhibitory effect was prevented by 2 mM EDTA (Fig. 9, upper panel). Ca2+ and Mg2+ slightly increased alk-SMase activity, but the activity was not significantly inhibited by EDTA (lower panel). We then further studied whether alk-SMase activity against SM could be inhibited by several inhibitors of NPPs. As shown in Fig. 10, imidazole dose-dependently inhibited alk-SMase activity against SM, 50% inhibition at 75 mM. At the same concentrations, imidazole had no effect on the activity of neutral SMase at equivalent catalytic capacity (upper panel). Orthovanadate, the inhibitor of ATPase and phosphatase, also dose-dependently inhibited alk-SMase activity (middle panel). When the enzyme was preincubated with ATP for 15 min at room temperature, the activity against SM was partly inhibited. ADP or AMP at the same concentrations did not show any inhibitory effects (bottom panel).

Because NPP2 has phospholipase D activity against lyso-PC (37), whether alk-SMase has similar activity was examined using [14C]palmitoyl lyso-PC as substrate. As shown in Fig. 11, the amount of lyso-PC was significantly decreased by alk-SMase, and [14C]monoacylglycerol was the only radioactive hydrolytic product found. The results indicate that human alk-SMase can also cleave the phosphocholine head group of lyso-PC, not the choline domain, and thus has phospholipase C and not phospholipase D activity.

DISCUSSION

Intestinal alk-SMase has been identified for more than 30 years (10). Our previous studies indicate that the enzyme is important for digestion of dietary SM and may have implications in colonic tumorigenesis and cholesterol absorption (15–18, 20, 24, 38). In this study by a combination of mass spectrometric analysis and Edman degradation, we identified a polypeptide structure corresponding to 415 residues from the purified enzyme. With the help of the information in the GenBank™ and a commercially available plasmid with a cDNA insert, we identified the full cDNA and amino acid sequences of human intestinal alk-SMase. By using a Myc tag linked to the cDNA, the validity of the cDNA for human alk-SMase was confirmed by high activity in the transfected cells, in anti-Myc immunoprecipitated proteins, and by the cross-reaction of anti-Myc-precipitated protein with anti-human alk-SMase antibody. The ability of the expressed enzyme to hydrolyze SM in vitro at optimal alkaline pH was also confirmed.

Although the enzyme was able to hydrolyze SM, its sequence shared no identity with cloned acid or neutral SMases (6, 7, 39). The result is reminiscent of our previous findings that alk-SMase has properties different from those of acid or neutral SMases (11, 21, 22), such as alkaline optimal pH, divalent ion independence, trypsin resistance, glutathione resistance, Triton X-100 inhibition, high thermostability, and specific bile salt dependence. BLAST search in GenBank™ demonstrated that the enzyme is related to the NPP family with 30–36% identities. The NPP family is a group of type II transmembrane ecto-nucleotidases with potentially important but still not fully characterized functions (40). Human alk-SMase shares the features of ecto-enzyme with the presence of a predicted signal peptide from outside to inside of the membrane, which may be cleaved. The active site of the enzyme is likely to be located outside the cells. The enzyme has an additional hydrophobic domain at the C terminus, which is a predicted signal anchor.
that may hook the enzyme to the plasma membrane. The enzyme is thus not spontaneously secreted, which may explain why the alk-SMase activity was increased in the medium of transfected COS-7 cells but to a smaller extent as compared with that in the cell extract. The finding fits our earlier result that the enzyme was difficult to be visualized using conventional immunostaining but was found to be located at the surface of the microvillar membrane of intestinal epithelial cells using the sensitive immunogold technique (22). It also fits our previous finding that bile and bile salts at physiological concentrations in the intestinal lumen may dissociate the alk-SMase from the enterocytes (41). As an ecto-enzyme, the main physiological substrate of intestinal alk-SMase may be the SM in the intestinal lumen and probably SM at the outer leaflet of the brush border membrane and not the intracellular SM pools. 

Although our transfection experiments with [3H]choline-labeled cells showing a decrease in SM that exceeded the resynthesis of SM by transferring phosphocholine from PC to ceram-ide, the physiological relevance of the results cannot be concluded at present. Detailed studies in stably transfected polarized brush border cells together with the identification of the location of the ceramide production are necessary to evaluate the physiological function of the enzyme in cells.


**TABLE I**

Comparison of alk-SMase with neutral and acid SMases

| Sample         | Assay condition | N-SMase | A-SMase | Ref. |
|----------------|-----------------|---------|---------|------|
| Alk-SMase      | 50 mM Tris, 0.15 mM NaCl, 1 mM Ca\(^{2+}\) | 7.4     | 4.5     | 8, 9 |
| Alk-SMase      | 50 mM Tris, 1 mM Mg\(^{2+}\) | 4.5     | Not dependent | 9    |
| Alk-SMase      | 50 mM Tris, 0.2 mM KCl, 1 mM Ca\(^{2+}\), pH 8.0 | 0.000   | 0.006 ± 0.001 | 47   |
| Alk-SMase      | 50 mM Tris, 0.15 mM NaCl, 2 mM Zn\(^{2+}\), pH 9.0 | 2.698 ± 0.431 | 0.473 ± 0.050 | 30   |
| Rat intestinal mucosa | 50 mM Tris, 0.15 mM NaCl, 1 mM Ca\(^{2+}\), pH 9.0 | 10.535 ± 3.292 | 1.850 ± 0.400 | 34   |

**TABLE II**

The activity of alk-SMase against pNMPP and pNPP

| Sample         | Assay condition | NPP activity |
|----------------|-----------------|--------------|
| Alk-SMase      | 50 mM Tris, 0.15 mM NaCl, 2 mM EDTA, 10 mM TC, pH 9.0 | 0.000, 0.000 ± 0.001 |
| Alk-SMase      | 50 mM Tris, 1 mM Ca\(^{2+}\), pH 9.0 | 0.000, 0.010 ± 0.002 |
| Alk-SMase      | 50 mM Tris, 0.2 mM KCl, 1 mM Ca\(^{2+}\), pH 8.0 | 0.000, 0.006 ± 0.002 |
| Alk-SMase      | 50 mM Tris, 0.15 mM NaCl, 2 mM Zn\(^{2+}\), pH 9.0 | 0.000, 0.003 ± 0.001 |
| Rat intestinal mucosa | 50 mM Tris, 0.15 mM NaCl, 1 mM Ca\(^{2+}\), pH 9.0 | 0.000, 0.006 ± 0.002 |
| Human colonic biopsy | 50 mM Tris, 0.2 mM KCl, 1 mM Ca\(^{2+}\), pH 8.0 | 0.000, 0.003 ± 0.001 |

Although alk-SMase is related to NPPs, attempts to identify the NPP activity of alk-SMase under different conditions were not successful. All NPPs have a catalytic residue threonine, which is contained in a motif TRTPPNH (the underlined T). In human alk-SMase, the catalytic residue is conserved. However, the motif sequence around the threonine has been considerably modified to TMTSPCH. It has been suggested that the motif sequence in NPPs, particularly the F adjacent to T is important for substrate specificity (31). This Phe is conserved in most NPPs but altered in alk-SMase, which may be crucial for differences in substrate specificity.

Even if alk-SMase did not have NPP activity, its activity against SM could be inhibited by factors that inhibit NPPs such as imidazole (32), which did not inhibit neutral SMase obtained from bacteria. Imidazole has been shown to stabilize a phosphoenzyme intermediate, leading to reduced catalytic rate (42).

**FIG. 9. Effects of divalent ions on purified alk-SMase activity.** The purified human alkaline SMase was diluted to 2.5 μg/ml. The activity in 5 μl of the sample (12.5 ng) was determined in the presence of different concentrations of Zn\(^{2+}\) and Mg\(^{2+}\). At zero concentration of divalent ions, 2 mM EDTA was added. The results are mean ± S.E. from three separate experiments.
Fig. 10. Effects of imidazole, orthovanadate, and ATP on purified alk-SMase activity. The purified human alkaline SMase was diluted to 2.5 µg/ml, and the neutral SMase from Bacillus cereus (Sigma) was diluted with 100 mM phosphate buffer, pH 7.4, to a level that is equally active as the diluted alk-SMase used. In the upper panel, the activity of 5 µl of alk-SMase (15.5 ng) or neutral SMase was determined in their optimal buffers (“Materials and Methods”) in the presence of different concentrations of imidazole. In the middle panel, 5 µl of the alk-SMase activity was assayed in the presence of different concentrations of orthovanadate. In the bottom panel, 5 µl of purified alk-SMase was first preincubated in 80 µl of assay buffer with different concentrations of ATP, ADP, or AMP for 15 min followed by adding 20 µl of [14C]SM to determine alk-SMase activity. The results are mean ± S.E. from three separate experiments.

Fig. 11. Hydrolytic activity of purified alk-SMase on lyso-PC. [14C]Palmitoyl-labeled lyso-PC (25 nmol) was incubated with 30 ng of alk-SMase (control) for 1 h. The lipids were isolated by phase partition and resolved by thin layer chromatography. The plates were developed with chloroform/methanol/acetic acid/water (50:40:3:0.6) and stained by iodine vapor. The bands of lyso-PC, lyso-PA, and monoglyceride (MG) were scraped according to the standards. The radioactivities in each band were determined and expressed as percentage of the total radioactivity in [14C]palmitoyl lyso-PC added. The results are mean ± S.E. from three separate experiments.

NPPs are metalloenzymes, and two metal-coordinating sites formed by 6 amino acid residues have been predicted recently (31). These amino acids are all conserved in human alk-SMase. However, alk-SMase activity is not dependent on Ca2+ or Mg2+ but abolished by Zn2+ at about 0.5 mM. The properties distinguish the alk-SMase from NPPs and from the SMase in the serum which is Zn2+-dependent (9, 49). The molecular mechanism underlying the differences is unknown. We hypothesize that although the catalytic residue and the coordinating metal sites of NPPs are conserved in alk-SMase, the modification of the motif in the active site could change the structure of the catalytic pocket, leading the metal-binding sites to favor interaction with the positively charged choline head group of SM (also PC and lyso-PC), rather than with divalent ions. Further studies are required to identify the binding mechanism of alk-SMase with its substrate and the mechanism of Zn2+-inhibition.

Northern blot analysis identified high mRNA levels of alk-SMase in human small intestine and liver, which is in agreement with our previous findings (35, 36, 47) that alk-SMase activity is present in human duodenum, small intestine, and bile. In this experiment, we were not able to identify mRNA of alk-SMase in the colon. Yet the alk-SMase clone (IMAGE 5186743) is derived from pooled materials of kidney, stomach, and colon, and our earlier studies (11, 15, 22, 35) have demonstrated the enzyme activity in colon but not in kidney and stomach. The expression of alk-SMase mRNA in colonic mucosa under normal and pathological conditions requires further investigation.

Alk-SMase may have important roles in the digestion of dietary SM. The finding that alk-SMase is an ecto-enzyme related to the NPP family opens new angles for searching other physiological functions. Recent studies have emphasized that the biological implications of ecto-enzymes are not restricted to the events outside of the cells. Ecto-enzyme can affect cell adhesion and organ development and generate signal molecules outside the cells, which in turn penetrate into the cells, affecting cellular functions and having impact on tumor metastasis (40). As a novel ecto-enzyme, alk-SMase may have broad physiological and pathological implications in the intestinal tract.

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