Intestinal alkaline sphingomyelinase hydrolyses and inactivates platelet-activating factor by a phospholipase C activity

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Alkaline sphingomyelinase (alk-SMase) is a new member of the NPP (nucleotide pyrophosphatase/phosphodiesterase) family that hydrolyses SM (sphingomyelin) to generate ceramide in the intestinal tract. The enzyme may protect the intestinal mucosa from inflammation and tumorigenesis. PAF (platelet-activating factor) is a pro-inflammatory phospholipid involved in pathogenesis of inflammatory bowel diseases. We examined whether alk-SMase can hydrolyse and inactivate PAF. [^{3}H]Octadecyl-labelled PAF was incubated with purified rat intestinal alk-SMase or recombinant human alk-SMase expressed in COS-7 cells. The hydrolytic products were assayed with TLC and MS. We found that alk-SMase cleaved the phosphocholine head group from PAF and generated 1-*O*-alkyl-2-acetyl-*sn*-glycerol. Differing from the activity against SM, the activity against PAF was optimal at pH 7.5, inhibited by EDTA and stimulated by 0.1–0.25 mM Zn^{2+}. The activity was abolished by site mutation of the predicted metal-binding sites that are conserved in all NPP members. Similar to the activity against SM, the activity against PAF was dependent on bile salt, particularly taurocholate and taurochenodeoxycholate. The V_{max} for PAF hydrolysis was 374 μmol $\cdot$ h^{-1} $\cdot$ (mg of protein)$^{-1}$. The hydrolysis of PAF and SM could be inhibited by the presence of SM and PAF respectively, the inhibition of PAF hydrolysis by SM being stronger. The PAF-induced MAPK (mitogen-activated protein kinase) activation and IL-8 (interleukin 8) release in HT-29 cells, and chemotaxis in leucocytes were abolished by alk-SMase treatment. In conclusion, alk-SMase hydrolyses and inactivates PAF by a phospholipase C activity. The finding reveals a novel function, by which alk-SMase may counteract the development of intestinal inflammation and colon cancer.

Key words: alkaline sphingomyelinase, colon cancer, inflammatory bowel disease, mitogen-activated protein kinase (MAPK), platelet-activating factor, phospholipase C.

INTRODUCTION

PAF (platelet-activating factor; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), first named by its platelet-aggregating activity, is a type of phospholipid with a phosphocholine head group at the position C-3 and an alkyl group linked to the glycerol by an ether bond. It is derived from 1-alkyl-2-acyl-glycero-3-phosphocholine in cell membrane, which is first hydrolysed by PLA_2 (phospholipase A_2) to lyso-PAF and subsequently acetified to PAF [1]. The catabolism of PAF is initiated by an acetyl hydrolase to form lyso-PAF, which is in turn either reacylated with long-chain fatty acid to yield 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine or hydrolysed by PLD (phospholipase D) [2]. PAF has been recognized as an important mediator in the pathogenesis of various intestinal diseases including IBD (inflammatory bowel disease), cholera and necrotizing enterocolitis in newborns [3–6]. The effects of PAF are mediated through G-protein-coupled receptors expressed in several tissues, including the intestinal mucosa [7], leading to functional changes of numerous signalling molecules such as activation of MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor κB), induction of biosynthesis of prostaglandins, expression of PLA_2 and release of a variety of cytokines, including IL-8 (interleukin 8), IL-1β and TNFα (tumour necrosis factor α) [8–11]. Increased PAF has been reported in faeces from patients with ulcerative colitis and Crohn's disease [12]. High levels of PAF were also found in colon cancer tissue [13].

Intestinal alk-SMase (alkaline sphingomyelinase) was first found in the intestinal content of human and the intestinal mucosa of rat and pig by Nilsson [14]. The enzyme was recently purified and cloned from both human and rat by our group and found to be a novel member of the NPP (nucleotide pyrophosphatase/phosphodiesterase) family, sharing no structural similarities with other SMases [15–17]. Although it belongs to the NPP family, the enzyme has no activity against nucleotide phosphodiesters, but was characterized as an SMase with some activity against phosphatidylcholine and lysophosphatidylcholine [17]. Alk-SMase is an ectoenzyme bound on the surface of intestinal microvilli with its C-terminal anchor, and is released to the intestinal lumen in an active form by pancreatic trypsin [18]. Previous studies showed that the physiological function of alk-SMase is to hydrolyse dietary SM (sphingomyelin), which is abundant in milk, egg, meat and fish, and also SM in the plasma membrane of epithelial cells [17]. Recent studies have shown decreased activity of the enzyme in the tissues of human long-standing ulcerative colitis and colonic adenoma and carcinomas, suggesting a potential role of the enzyme in both colonic inflammation and carcinogenesis [19–21]. An alternative splicing of alk-SMase mRNA, which leads to inactivation of the enzyme, has been found in colon cancer HT-29 cells [22].

Both PAF and SM are phospholipids with a positively charged phosphocholine head group and a hydrophobic tail. Previous studies did not find any type of SMase that can hydrolyse PAF;

Abbreviations used: AAG, 1-*O*-alkyl-2-acetyl-*sn*-glycerol; SMase, sphingomyelinase; alk-SMase, alkaline SMase; CMC, critical micelle concentration; DG, diacylglycerol; DTT, dithiothreitol; FCS, foetal calf serum; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; IL, interleukin; LC-MS, liquid chromatography MS; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NPP, nucleotide pyrophosphatase/phosphodiesterase; PAF, platelet-activating factor; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; SM, sphingomyelin; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate, TNFα, tumour necrosis factor α.

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only an enzyme originally predicted to be a neutral SMase was found to hydrolyse lyso-PAF but not PAF by a PLC activity [23]. Whether alk-SMase can hydrolyse PAF, which is coexisting with the enzyme in the intestinal lumen in the inflamed bowel, has not been investigated. Our study identified a novel function of intestinal alk-SMase to hydrolyse and inactivate PAF. The finding supports the potential protective effects of the enzyme against IBDs and colon cancer.

MATERIALS AND METHODS

Materials

[^{3}H]Octadecyl-labelled PAF (1-*O*-[^{3}H]octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), anti-Myc antibody, HRP (horseradish peroxidase)-conjugated anti-mouse IgG antibody and the ECL® Advance Western Blotting kit were purchased from Amersham Biosciences (Uppsala, Sweden). Specific radioactivity of the labelled PAF was 311 mCi/mg and was diluted with unlabelled PAF to 200 d.p.m./pmol. SM was purified from bovine milk and labelled with [^{14}C-CH_3]choline ([^{14}C]SM) by Dr Peter Ström (Astra Zeneca, Stockholm, Sweden). The specific radioactivity of the labelled SM was 56 μCi/mg. The plasmids for wild-type human alk-SMase expression were constructed as described previously [17,24]. Rat intestinal alk-SMase was purified from rat intestine in our laboratory [15]. Lipofectamine™ 2000 and cell culture medium were obtained from Invitrogen (Paisley, U.K.). Anti-MAPK (phospho-specific) antibody was from Calbiochem (Darmstadt, Germany). Goat anti-actin antibody was from SDS Biosciences (Falkenberg, Sweden) and rabbit anti-goat IgG–HRP conjugate was from Zymed (San Francisco, CA, U.S.A.). Si 60F TLC plates were from Merck (Darmstadt, Germany). The human IL-8 immunoassay kit was purchased from R&D Systems (Abingdon, Oxfordshire, U.K.). Unlabelled PAF, lyso-PAF, bacterial neutral SMase and other chemical agents used were purchased from Sigma (Stockholm, Sweden).

Determination of the hydrolytic products of PAF by TLC and MS

Rat intestinal alk-SMase (5 μl), which contained 1.5 ng of protein, was mixed with 100 pmol of [^{3}H]PAF in 95 μl of 50 mM Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.25 mM $ZnCl_2$ and 6 mM TC (taurocholate) (P buffer, optimized by preliminary experiment), and incubated at 37 °C for 2 h. The reaction was terminated by adding 0.4 ml of chloroform/methanol (2:1, v/v), followed by centrifugation at 9000 ***g*** for 10 s. The lower phase containing the lipids was dried under nitrogen, dissolved in 20 μl of chloroform/methanol (2:1, v/v) and loaded on Si 60F TLC plates for TLC. Unlabelled PAF and DG (diacylglycerol) were loaded as standards. The plates were developed in chloroform/methanol/ammonia (65:25:4, by vol.) and stained with iodine vapour. Each lane was cut into 17 fractions and each fraction was scraped for liquid-scintillation counting.

To further identify the hydrolytic products, 5 nmol of unlabelled PAF was incubated with 25 μl of rat intestinal alk-SMase in 475 μl of P buffer at 37 °C for 2 h. The reaction was terminated as above and the lower phase containing the lipids was dried under nitrogen and dissolved in 25 μl of ethanol for LC-MS (liquid chromatography MS) assay. Aliquots of 5 μl of the lipid extract were injected with flow injections into a PerkinElmer Series 200 LC system with an autosampler (Applied Biosystems, Norfolk, CT, U.S.A.), coupled with an API 3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, ON, Canada) with a Turbo electrospray ion source operated in positive ion mode at 375 °C. The mass spectrometer was operated in the single quadrupole mode in the range *m*/*z* 200–800. The declustering potential was 30 V and the ion spray 4200 V.

Assay of SMase activity

Neutral SMase and alk-SMase were determined in different buffers as described previously [25]. For the alk-SMase assay, the buffer was 50 mM Tris/HCl buffer (pH 9.0) containing 0.15 M NaCl, 2 mM EDTA and 6 mM TC (K buffer), and for neutral SMase, the buffer was 50 mM Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl, 4 mM Mg^{2+} and 0.12 % Triton X-100 (N buffer). For characterizing the effect of Zn^{2+}, the SM hydrolytic activity was also determined in P buffer with and without Zn^{2+}. For each determination, 5 μl of the enzyme was incubated with 100 pmol of [^{14}C]SM in different buffers to a final volume of 100 μl. After incubation 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 9000 ***g*** for 10 s. An aliquot (100 μl) of the upper phase containing the cleaved phosphocholine was analysed for radioactivity by liquid scintillation.

Comparison of PAF hydrolytic activities of rat alk-SMase and bacterial neutral SMase

Bacterial neutral SMase was first diluted in PBS to a concentration equivalent to that of rat alk-SMase. Rat intestinal alk-SMase or bacterial neutral SMase was incubated with 100 pmol of [^{3}H]PAF in P buffer and the production of AAG (1-*O*-alkyl-2-acetyl-*sn*-glycerol) was assayed as described above. The activity against PAF was also assayed in K buffer and N buffer, which are optimal for alk-SMase and neutral SMase against SM respectively.

Expression of human alk-SMase in COS-7 cells

Transient expression of alk-SMase in COS-7 cells was performed as described previously [17]. The medium of the cell culture was collected 48 h after transfection, 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 DTT (dithiothreitol), 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM TC, followed by sonication for 10 s. After centrifugation at 12000 ***g*** for 10 min at 4 °C, the cell-free extract was collected. The activities against PAF and SM in the medium and cell-free extracts were assayed in P and K buffers respectively.

Characterization of the activity against PAF

To determine the hydrolytic capacity of rat alk-SMase against PAF, 80–40000 pmol of [^{3}H]PAF was mixed with purified rat intestinal alk-SMase (1 ng) in P buffer and incubated at 37 °C for 1 h. Lineweaver–Burk plot was performed, and V_{max} (the maximal rate) and K_m (Michaelis constant) were determined. The hydrolytic capacity of the enzyme against PAF in the presence of SM was determined by mixing 100 pmol of PAF with SM ranging from 100 to 10000 pmol in P buffer. A similar study was also carried out to look at the influence of PAF on SM hydrolysis in K buffer by alk-SMase.

To identify the optimal pH of alk-SMase against PAF, 100 pmol of [^{3}H]PAF was incubated with the enzyme in 50 mM Tris/maleate buffers with pH values from 5 to 7 and in 50 mM Tris/HCl buffers with pH from 7 to 10.0, containing 0.15 M NaCl, 0.25 mM $ZnCl_2$ and 6 mM TC at 37 °C for 60 min. The produced AAG was assayed by TLC as described above.

Since the activity of alk-SMase against SM is dependent on bile salts and the maximal stimulatory effects occur at the CMCs (critical micelle concentrations) [15], we studied whether bile salts also affect its activity against PAF. The PAF hydrolytic activity was determined in P buffer in the presence of different bile salts. The concentration-dependent effects were performed for TC and TDC (taurodeoxycholate) and the efficacy of different bile salts

was compared at the CMC as described in [15]. The CMCs for bile salts examined were: 6 mM for TC, glycocholate, cholate and Chaps, 1.5 mM for TDC and TCDC (taurochenodeoxycholate) and 0.12 % for Triton X-100.

Effect of metal ions and importance of the predicted metal-binding sites on the activity against PAF

Alk-SMase is related to the NPP family. A general property of NPPs is the dependency of the activity on metal ions, particularly Zn^{2+}. There are six amino acid residues forming two predicted metal-binding sites in this enzyme family, which were conserved also in alk-SMase [17,26]. An interesting finding that reveals a unique property of alk-SMase is that the activity against SM is not dependent on metal ions, but is inhibited by Zn^{2+} and also by mutations within the metal-binding site. The question whether the novel activity of the enzyme against PAF is affected by metal ions and by the metal-binding sites was addressed. We firstly determined the activity in Tris/HCl buffer containing 0.15 M NaCl and 6 mM TC (pH 7.5) in the presence of either 2 mM EDTA or different concentrations of Zn^{2+}, Ca^{2+} or Mg^{2+}. Secondly, we measured the activity after site-directed mutations of the metal-binding sites. The mutagenesis was carried out by the megaprimer PCR method [27]. The oligonucleotide for D199A (antisense) was 5′-cgtacctgtggcccgtggaggccggctccccgaagtagagtg-3′ and that for D246A (sense) was 5′-gcctcaacctgatcatcacatccgcccacggcatgacgaccgtggacaaac-3′, in which the altered codons are underlined. The sense and antisense primers for alk-SMase were 5′-tcggtaccgaaagcatgagaggcccggccgtcctc-3 and 5′-tagcggccgcctgcgacctcagacagaagaat-3′. The wild-type alk-SMase cDNA was used as a template. The mutated genes were cloned into the expression vector pcDNA4/TO/Myc-His B at KpnI and NotI sites as the wild-type gene. The constructed plasmids with wild-type or mutated alk-SMase were transferred in COS-7 cells and the PAF hydrolytic activity in cell-free extraction was measured. To control the expression rates, Western-blot analysis was performed, using anti-Myc antibody (1:5000) as the first antibody and anti-mouse IgG antibody (1:50 000) conjugated with HRP as the second.

Functional studies of PAF after alk-SMase treatment

To study the functional changes of PAF after treatment with alk-SMase, 100 pmol of PAF was incubated with or without (control) 1.5 ng of rat intestinal alk-SMase in P buffer at 37 °C for 2 h. The reaction was terminated as described above. After phase partitioning, the upper phase was withdrawn and the lower phase was dried under nitrogen. The dried PAF in the lower phase was dissolved in PBS buffer with 0.25 % BSA and used for functional studies as follows.

Chemotaxis study

Human promyelocyte HL-60 cells grown in RPMI 1640 supplemented with 10 % (v/v) FCS (foetal calf serum) and 1 % penicillin/streptomycin were washed and incubated in chemotaxis medium (RPMI 1640 supplemented with 0.5 % BSA for 1.5 h at 37 °C prior to use). For chemotaxis assays, HL-60 cells (2×10^5 cells/well in 100 μl of chemotaxis medium) were added to the top inserts of 12-well Transwell plates (5 μm pore size; Corning, Corning, NY, U.S.A.). PAF or PAF treated with alk-SMase (600 μl) was added to the bottom well and the plates were placed in a 37 °C incubator. After 90 min, medium was removed from the bottom well and the migrating cells were enumerated and phenotyped by flow cytometry (FACSCalibur; BD Biosciences). The percentage of $CD11b^+$ cells in the starting and migrating population was determined after staining with anti-CD11b antibody (M1/70; Pharmingen, U.S.A.) and streptavidin-allophycocyanin (Pharmingen, U.S.A.). Dead cells were excluded from the analysis using 7-amino-actinomycin D (Sigma–Aldrich). The percentage of migrating $CD11b^+$ cells was determined by the equation: [(number of $CD11b^+$ cells in the bottom well)/(number of $CD11b^+$ cells added to the top well)] $\times$ 100.

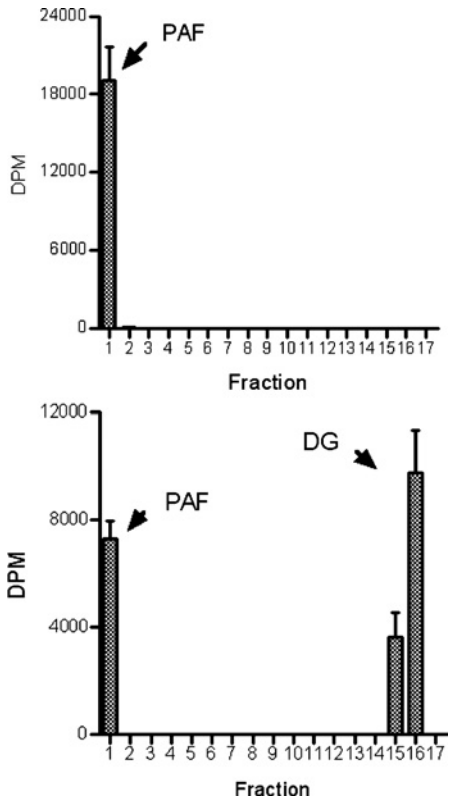

Figure 1 Identification of hydrolytic products of PAF by TLC

[^{3}H]PAF was incubated without (upper panel) or with rat intestinal alk-SMase (bottom panel) in 50 mM Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.25 mM $ZnCl_2$ and 6 mM TC (P buffer) at 37 °C for 2 h. Lipids were extracted, separated by TLC and stained with iodine vapour. Authentic PAF and DG were used as standards. After TLC, each lane was divided into 18 fractions and each fraction was scraped, and the radioactivity was assayed by liquid scintillation. Results shown are means ± S.E.M. for three separate experiments.

MAPK assay

The activation of MAPK induced by PAF was examined in HT-29 cells, which were cultured in monolayer in RPMI 1640 with 2 mM L-glutamine, 10 % FCS and 100 i.u. (international units)/ml penicillin and 100 μg/ml streptomycin on 6-well plates as described in [22]. Before stimulation, the cells were adapted for serum-free medium for 2 h, followed by adding PAF and alk-SMase-treated PAF to a final concentration of 100 pmol/ml. After 5 min, the cells were lysed in lysis buffer containing 20 mM Tris/HCl, 150 mM NaCl, 1 % Triton X-100, 1 % Nonidet P40, 2 mM EDTA, 0.4 mM $(NH_4)_6Mo_7O_{24}$, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM aprotinin, 10 μg/ml leupeptin, 10 mM $Na_4P_2O_7$ and 1 mM DTT (pH 7.5) [28]. The cell-free extracts (50 μg of protein) were subjected to Western blotting for phosphorylated MAPK using anti-phospho-MAPK antibody (1:5000) and anti-mouse IgG antibody (1:50 000) conjugated with HRP. The phosphorylated p42 and p44 MAPKs were identified by ECL® advance reagent. The remitted light was recorded on a Kodak X-ray film. The membranes were then stripped and reblotted with goat anti-actin (1:1000) as a loading control.

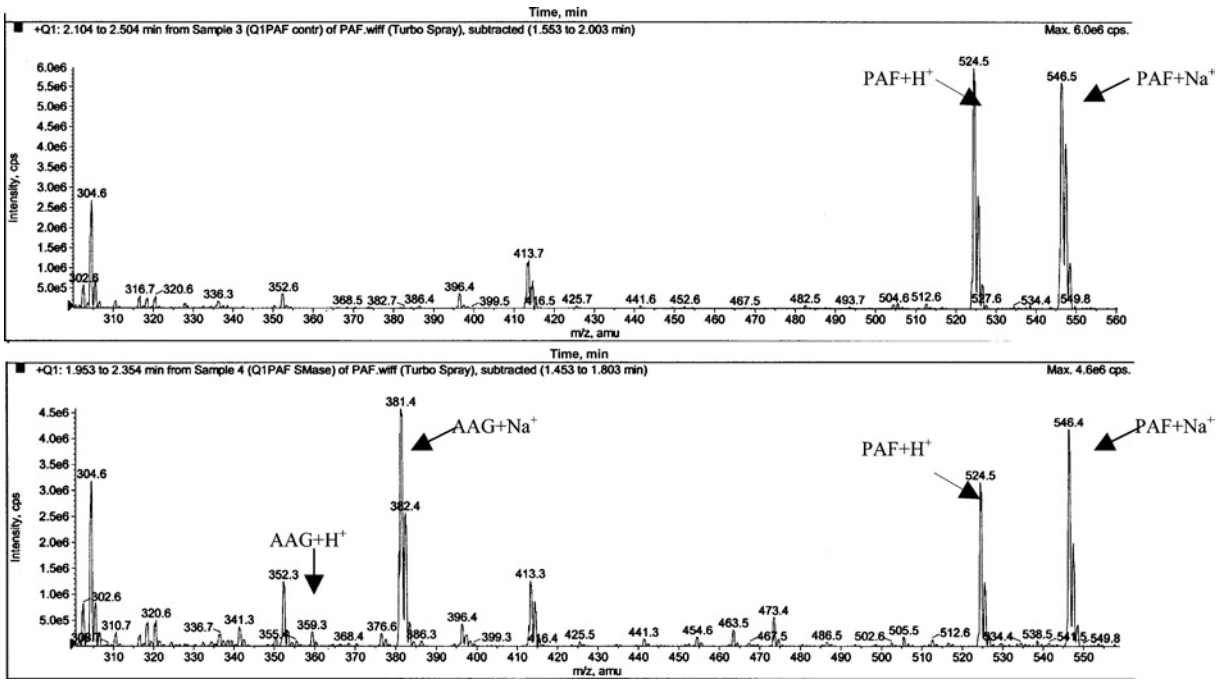

Figure 2 Identification of hydrolytic product of PAF by MS

PAF (5 nmol) was treated with 1.5 ng of rat intestinal alk-SMase (lower panel) or without (upper panel). The hydrolytic products were extracted and subjected to LC-MS. The *m*/*z* values 524.5 and 546.5 in the Figure are consistent with the protonated ion mass and sodium ion mass of PAF, and 381.4 and 359.3 are consistent with the sodium ion mass and protonated ion mass of AAG.

Assay of IL-8 release

For determining the PAF-induced release of IL-8, HT-29 cells were subcultured in 96-well plates. After attachment overnight, the cells were incubated with PAF or alk-SMase-treated PAF (100 pmol/ml) for 16 h. The levels of IL-8 in the medium were analysed by an IL-8 ELISA kit obtained from R&D Systems. The assay procedure followed the instructions of the manufacturer.

RESULTS

Rat intestinal alk-SMase hydrolysed PAF

Rat alk-SMase was incubated with [^{3}H]PAF and the hydrolytic products were examined by TLC. The whole lane was divided into 18 fractions and the radioactivity in each fraction was determined. As shown in Figure 1 (upper panel), without alk-SMase treatment, only one radioactive peak corresponding to PAF was identified. The results indicate that the spontaneous hydrolysis of PAF was negligible under the conditions. Treating the PAF sample with alk-SMase (lower panel) significantly reduced the radioactivity of the PAF peak from $19\,100 \pm 2305$ to 7203 ± 592 d.p.m. ($P < 0.01$), and induced a radioactive neutral lipid portion in fractions 15 and 16, which migrated similarly to DG. No radioactivity was found in other fractions.

To characterize the neutral lipid portion induced by alk-SMase treatment from PAF, the samples were further analysed by LC-MS. As shown in Figure 2 (upper panel), the predominant mass values seen in the control sample were 524.5 and 546.5, which represent the protonated and sodium ion mass values of PAF respectively [29]. In the alk-SMase-treated sample (lower panel), a predominant mass of 381.4 and a minor mass of 359.3 were identified, which were consistent with the mass of sodium and protonated ions of 1-*O*-palmitoyl-2-acetyl-*sn*-glycerol respectively. Meanwhile, the intensity of protonated PAF (*m*/*z* 524.5) and sodium PAF (*m*/*z* 546.4) peaks decreased. No mass corresponding to 1-*O*-palmitoyl-2-acetyl-glycerol-phosphatic acid (*m*/*z* 438 for its protonated ion or 460 for its sodium ion) or 1-*O*-hexadecyl-glycerol-phosphocholine (*m*/*z* 482 for its protonated ion or 504 for its sodium ion) was found. Thus PAF was hydrolysed by alk-SMase at the phosphodiester bond to generate AAG and phosphocholine. No PLD or PAF acetylhydrolase activity was identified. We also treated lyso-PAF with alk-SMase in a similar way and only found a minor mass peak of 339.3, which is consistent with the sodium ion mass of 1-*O*-palmitoyl-glycerol, but could hardly see any changes in the intensity of lyso-PAF mass peaks (482.4 and 502.4; results not shown). The results suggest that alk-SMase has some PLC activity against lyso-PAF, but the activity is much smaller than that for PAF.

Activity of human alk-SMase expressed in COS-7 cells

To confirm further that the activity against PAF is derived from alk-SMase, human alk-SMase cDNA was expressed in COS-7 cells and the activities against both PAF and SM in the cell-free extracts and culture medium were determined. As shown in Figure 3, expression of alk-SMase cDNA significantly increased the activity against PAF in the cell lysate (Figure 3A) and in cell medium (Figure 3C). The increased activity against PAF was parallel to that against SM (Figures 3B and 3D).

Comparison of the activities against SM and PAF

To compare the activity against PAF and SM, both P and K buffers were used. When assayed in P buffer, the activity against PAF was approx. 9.9 ± 0.9 % of its activity against SM and when assayed in K buffer, 4.3 ± 1.1 %. Little activity of neutral SMase against PAF (< 0.1 %) was found in either P buffer or N buffer compared with its activity against SM (Figure 4). The N buffer is optimal for neutral SMase against SM.

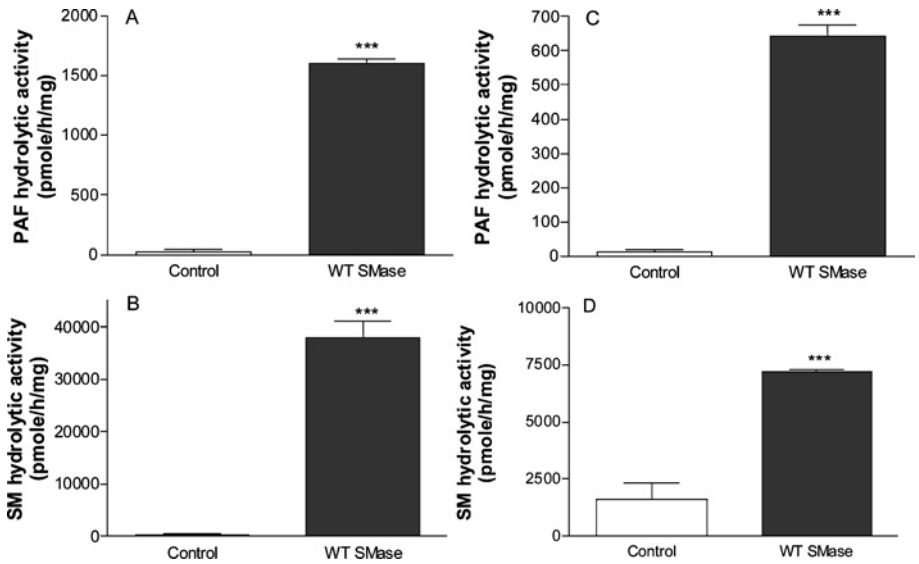

Figure 3 PAF hydrolytic activity in COS-7 cells transfected with human alk-SMase cDNA

COS-7 cells were transfected with human alk-SMase cDNA in the presence of Lipofectamine™ 2000, followed by incubation of the cells for 48 h. The medium was collected and the cell-free extracts were prepared. The activity against PAF and SM was determined. (**A**, **B**) The activity against PAF and SM in the cell-free extracts. (**C**, **D**) The activity against PAF and SM in culture medium. Results shown are means ± S.E.M. for three separate experiments. ***$P < 0.001$ compared with control.

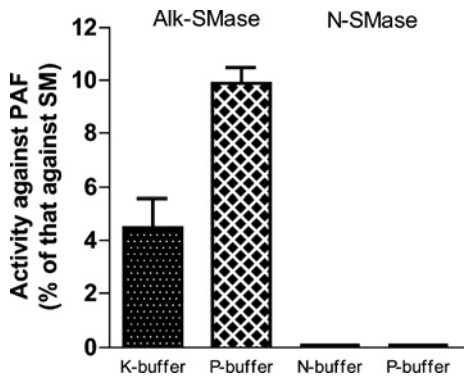

Figure 4 Comparison of the effects of alk-SMase and neutral SMase

Both rat alk-SMase and bacterial neutral SMase were diluted to equivalent hydrolytic capacity and incubated with PAF in different buffers. P buffer is optimal for the activity against PAF; K and N buffers are optimal for alk-SMase and neutral SMase against SM respectively as described in the Materials and methods section. The formation of AAG was analysed by TLC. Results shown are means ± S.E.M. for three separate experiments.

The hydrolysis of PAF by alk-SMase in the presence of SM is shown in Figure 5 (upper panel). No significant inhibition of PAF hydrolysis was observed when the amounts of SM and PAF were equal (0.1 nmol). However, higher amount of SM inhibited the hydrolysis of PAF, with 90 % inhibition being observed when SM is 100 times higher than PAF. PAF also inhibited SM hydrolysis, but the inhibition was weaker and higher concentrations were required as compared with the inhibitory effects of SM on PAF hydrolysis (Figure 5, lower panel).

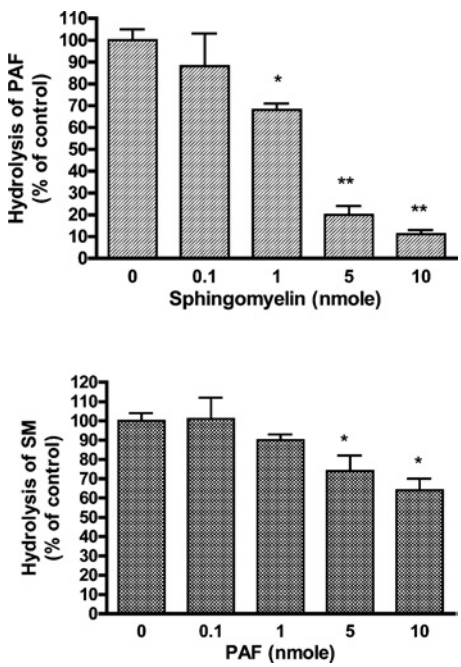

Figure 5 Competition between PAF and SM for hydrolysis by alk-SMase

In the upper panel, PAF hydrolysis by alk-SMase (1.5 ng) was determined in P buffer containing 100 pmol of PAF and different amounts of SM. After a 2 h incubation, the lipids were extracted and the hydrolytic products were separated by TLC. The radioactivities of AAG were assayed by liquid scintillation. In the lower panel, the activity against SM was determined in the K buffer containing 100 pmol of [^{14}C]SM and different amounts of PAF. After incubation 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 9000 ***g*** for 10 s. An aliquot (100 μl) of the upper phase containing the cleaved phosphocholine was analysed for radioactivity by liquid scintillation. Results shown are means ± S.E.M. for three separate experiments. *$P < 0.05$ and **$P < 0.01$.

Characterization of the PAF hydrolytic activity of rat intestinal alk-SMase

Characterization of the hydrolytic activity against PAF was performed with purified rat intestinal alk-SMase. The activity against PAF was demonstrated at pH from 6 to 9 and the maximal hydrolytic activity was found at pH 7.5 (Figure 6, top panels). The time and dose dependency of the activity is shown in the top right and bottom left panels respectively. Under the assay conditions where 1.5 ng of rat intestinal alk-SMase was incubated

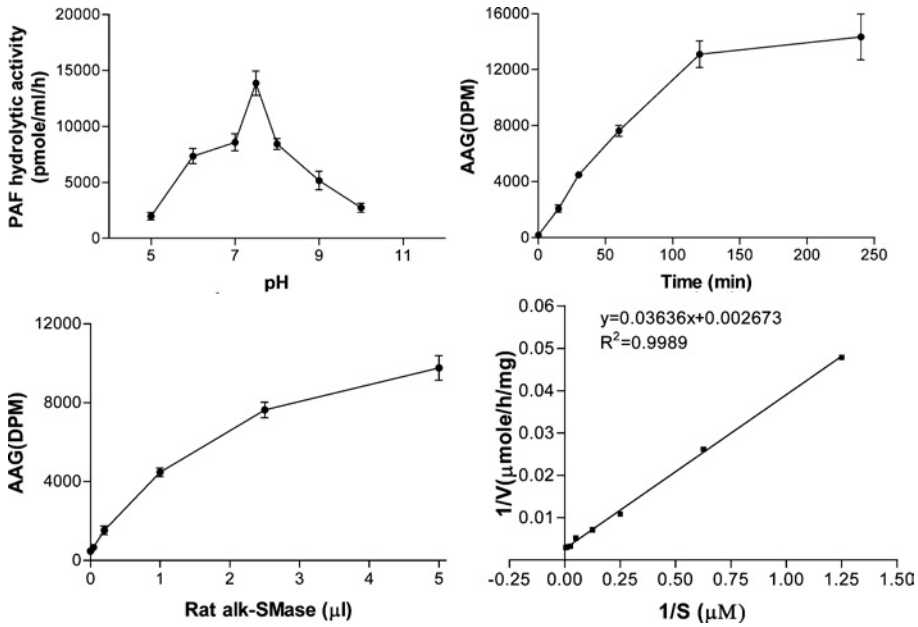

Figure 6 Characterization of the PAF hydrolytic effects of alk-SMase

In the top left panel, 1.5 ng of rat intestinal alk-SMase was incubated with 100 pmol of [^{3}H]PAF in 50 mM Tris/maleate buffer at pH 5–7 or Tris/HCl buffer at pH 7.5–9.0, containing 0.15 M NaCl, 0.25 mM $ZnCl_2$ and 6 mM TC, at 37 °C for 1 h. In the top right panel, rat intestinal alk-SMase was incubated with 100 pmol of [^{3}H]PAF in P buffer at 37 °C and the reaction was terminated at different times. In the bottom left panel, 0–3 ng of alk-SMase was incubated with 100 pmol of [^{3}H]PAF at 37 °C for 60 min. The production of AAG was separated with TLC and radioactivity was analysed by liquid scintillation. Results shown are means ± S.E.M. for three separate experiments. In the bottom right panel, 1 ng of purified rat alk-SMase was incubated with different amounts of [^{3}H]PAF ranging from 80 to 40 000 pmol at 37 °C for 60 min. The production of AAG was analysed and the hydrolytic capacity was determined by Lineweaver–Burk plot. A similar Figure was obtained from another experiment.

with 100 pmol of [^{3}H]PAF, the generation of AAG from PAF increased with the incubation time linearly up to 2 h. At this time point, approx. 70 % of total PAF was hydrolysed. The bottom panels show that the activity of alk-SMase against PAF was dose-dependent. Based on the results described above, we incubated the enzyme with different amounts of PAF in P buffer for 1 h and present the results as a Lineweaver–Burk plot. The V_{max} and K_m under the conditions used were approx. 374 μmol · h^{-1} · mg^{-1} and 13.6 μM respectively.

We previously showed that bile salts, particularly TC and TCDC, stimulated alk-SMase activity with the maximal effect occurring around the CMCs [15]. Similar results were obtained for the activity against PAF. As shown in Figure 7 (upper panel), the PAF hydrolytic activity was dose-dependently increased by both TC and TDC and the maximal effect occurred at CMC (6 and 1.5 mM respectively). We compared the efficiency of different bile salts at their respective CMCs and also examined the effects of two widely used synthetic detergents, CHAPS and Triton X-100. As shown in the bottom panel, among these bile salts, TC and TCDC were much more effective than other bile salts tested. The non-physiological detergent Triton X-100 and CHAPS did not show any stimulated effects at all. The results are well in agreement with previous characterization of the activity against SM [15].

Alk-SMase belongs to the NPP family and a common property of most NPP members is the dependency of the activity on metal ions, particularly Zn^{2+} [26]. However, the activity of alk-SMase against SM is not dependent on metal ions and is inhibited by Zn^{2+} and by mutations of the metal-binding sites under the optimal conditions [24]. We therefore examined the effects of metal ions on the activity against PAF. We found that, in contrast with the activity against SM, the activity against PAF does depend on metal ions. As shown in Figure 8, EDTA at 2 mM inhibited the

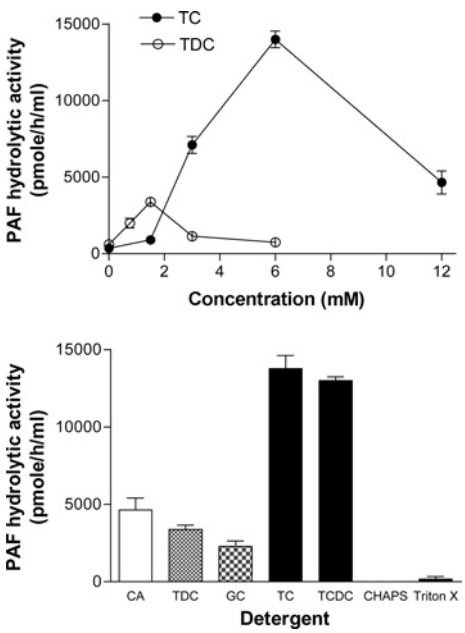

Figure 7 Effect of bile salts on PAF hydrolytic activity of alk-SMase

In the upper panel, the PAF hydrolytic activity was assayed in P buffer containing different concentrations of TC and TDC. In the bottom panel, the activity was assayed in the same buffer with different bile salts and synthetic detergents at CMCs: 6 mM for cholate (CA), glycocholate (GC), TC and CHAPS, 1.5 mM for TDC and TCDC and 0.12 % for Triton X-100 in the presence of 100 pmol of [^{3}H]PAF. Results shown are mean ± S.E.M. for three separate experiments.

activity by 58 % (top left panel) and the activity was increased with Zn^{2+} in low concentrations. However, when the concentration of Zn^{2+} was higher than 0.25 mM, the activity against PAF

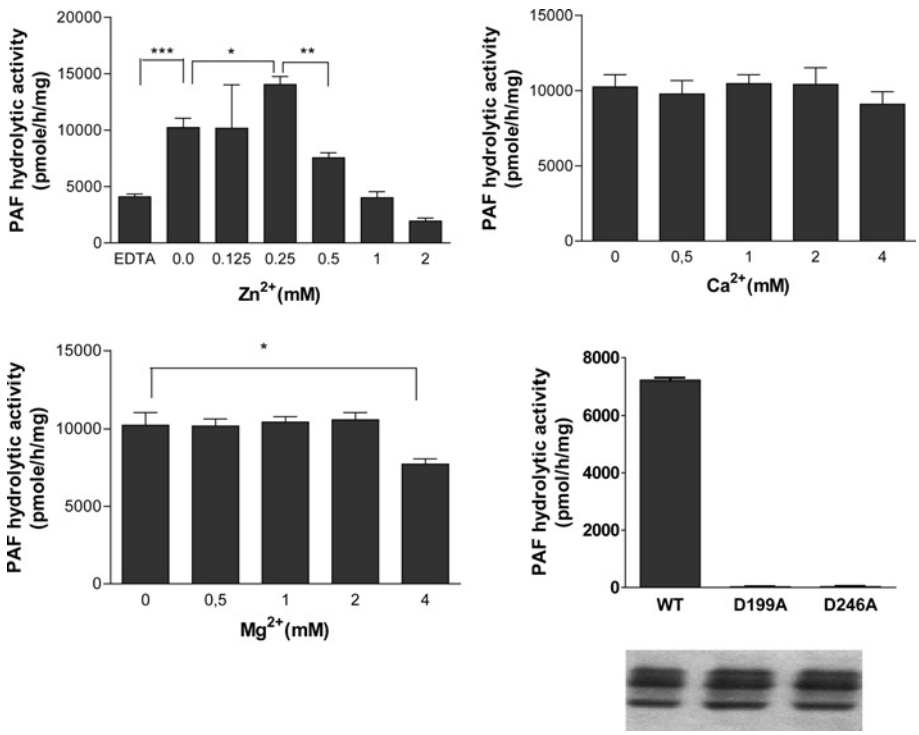

Figure 8 Effect of metal ion on PAF hydrolytic activity of alk-SMase

The PAF hydrolytic activities of purified rat alk-SMase were assayed in 50 mM Tris/HCl (pH 7.5), with 100 pmol of [^{3}H]PAF, 0.15 M NaCl, 6 mM TC and 2 mM EDTA in the presence of 0–2 mM Zn^{2+} (top left panel) 0–4 mM Ca^{2+} (top right panel), or 0–4 mM Mg^{2+} (bottom left panel). Bottom right panel: COS-7 cells were transfected with wild-type human alk-SMase cDNA (WT), or with D199A or D246A mutant cDNA. After expression for 48 h, the cells were lysed and the PAF hydrolytic activity in cell-free extraction was assayed in P buffer and the SM hydrolytic activity was assayed in K buffer. Western blotting for alk-SMase is shown under the panel. All the results shown are means $\pm$ S.E.M. for three separate experiments. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared with the one indicated.

was inhibited in a dose-dependent manner, like the activity against SM [17]. Ca^{2+} had little effect on the activity against PAF and Mg^{2+} showed a mild inhibitory effect. Asp^{199} and Asp^{246} are two amino acid residues that participate in the formation of the two putative metal ion-binding sites in alk-SMase and NPP [17,26]. These sites are important for activity against PAF in the presence of 0.25 mM Zn^{2+}, as shown in the bottom panel of Figure 8. D199A or D246A mutation abolished the enzyme activity against PAF, whereas the expression rates of the enzyme in the host cells were compatible (Figure 8, bottom panel). The three bands shown in the Western blot represent different glycosylation forms of the enzyme as recently reported [24].

Our previous study showed that Zn^{2+} inhibited alk-SMase activity against SM [17]. In the present study, we further examined if Zn^{2+} also inhibited SM hydrolysis under the conditions optimal for its activity against PAF. The activity was assayed in P buffer with and without Zn^{2+}, in comparison with the activity exhibited in K buffer. We found that SM hydrolysis in the P buffer without Zn^{2+} and with Zn^{2+} (0.25 mM) were approx. 74 ± 11 and 65 ± 8 % of that in K buffer respectively. The results indicate that the inhibitory effect of Zn^{2+} on SM hydrolysis is weaker under the conditions optimal for hydrolysing PAF.

Functional changes of PAF by alk-SMase

Finally, we studied whether hydrolysis of PAF by alk-SMase would affect the functions of PAF using three approaches: the changes in PAF's ability to induce chemotaxis in leucocytes, to activate MAPK and to induce IL-8 production by colonic cells. PAF was pretreated with alk-SMase, isolated, and re-dissolved in a BSA-containing buffer. Pretreatment of PAF with the enzyme completely blocked PAF's ability to induce HL-60 cell chemotaxis (Figure 9, top panel). As shown in the middle panel of Figure 9, PAF at 100 nM increased phosphorylation of both p42 and p44 MAPKs in HT-29 cells. The effect was inhibited by preincubation of the PAF with alk-SMase. The lower panel shows that PAF at the same concentration that activated MAPK increased IL-8 release from HT-29 cells by 23 % ($P < 0.05$). This effect was also abolished by alk-SMase treatment.

DISCUSSION

The present study demonstrates that intestinal alk-SMase can hydrolyse and inactivate PAF, a well-known lipid mediator involved in pathogenesis of several inflammatory diseases including IBD [6]. The activity was identified with highly purified rat intestinal alk-SMase and with the recombinant human enzyme expressed in COS-7 cells. The activity is characterized as a type of PLC, since the enzyme generates a neutral lipid portion similar to DG in TLC and this neutral lipid was further identified to be AAG by LC-MS. No formation of lyso-PAF or 1-*O*-alkyl-2-acetyl-glycero-phosphatidic acid was found. The finding is novel as no known SMase has been shown to hydrolyse PAF by a PLC activity.

When the activity was characterized and compared with its activity against SM, several similarities and differences were identified. A major similarity is the bile salt dependency. The activity against PAF is also stimulated by bile salt, particularly by TC and TCDC, while other detergents such as Triton X-100 and CHAPS had no effect. The dependency of alk-SMase on specific bile salts is a unique property of the enzyme, which allows the

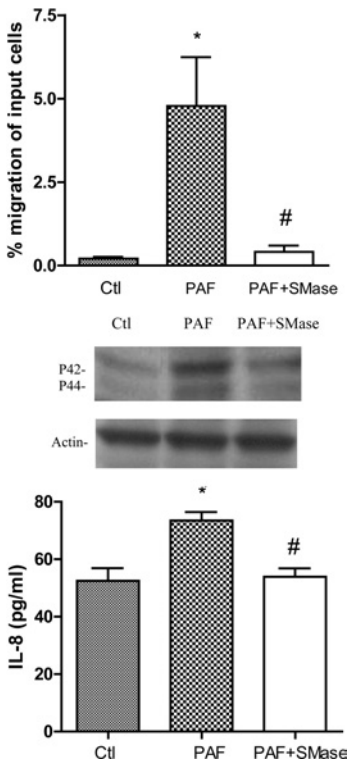

Figure 9 Effect of intestinal alk-SMase on PAF-induced chemotaxis, MAPK activation and IL-8 release

PAF was preincubated with or without rat intestinal alk-SMase for 2 h, then extracted and dissolved in PBS with 0.25 % BSA. In the top panel, HL-60 cells were stimulated with 1 nM PAF treated with or without alk-SMase. The chemotaxis of the cells was determined. In the middle panel, 100 nM PAF treated with or without alk-SMase was incubated with HT-29 cells for 5 min, and the phosphorylated MAPK was determined by Western-blot analysis. The membrane was stripped and reblotted with anti-actin antibody as a loading control. In the lower panel, PAF treated with or without alk-SMase was incubated with HT-29 cells in a 96-well plate at the concentration of 100 nM for 16 h. The release of IL-8 was assayed by an IL-8 ELISA kit. Results shown are means ± S.E.M. for three separate experiments. $*P < 0.05$ compared with control; $\#P < 0.05$ compared with PAF.

enzyme to exert its function against both SM and PAF in the intestinal environment. It may also be the reason why previous studies did not find PAF PLC activity in the intestine when measured in a bile-salt-free buffer [30]. Characterization also showed two major differences. The first is the optimal pH, which is 7.5 and is lower than that for the activity against SM, but closer to the pH in the intestinal lumen. The second is the zinc dependency. We previously found that the activity of alk-SMase against SM was inhibited by Zn^{2+}. A 60 % inhibition was observed with 0.25 mM Zn^{2+} [17]. Interestingly, we found in the present study that its activity against PAF is increased by Zn^{2+} at 0.25 mM and inhibited by EDTA. The Zn^{2+} dependency resembles that of other NPP members [26,31]. However, the activity of alk-SMase against PAF could also be inhibited by Zn^{2+} at concentrations higher than 0.25 mM and by mutations of the metal-binding sites, similar to its activity against SM. Under optimal conditions for the activity of alk-SMase against PAF, the inhibitory effect of Zn^{2+} on SM hydrolysis was found to be much weaker than under the conditions optimal for SM hydrolysis. Thus, at neutral pH and in the presence of Zn^{2+} in the gut, both SM and PAF may be hydrolysed. Although high concentrations of SM inhibit PAF hydrolysis, hydrolysis of SM by alk-SMase may suppress the inhibition and thus facilitate its second activity for PAF hydrolysis. The conclusion is that the metal-binding sites are necessary for the hydrolysis of both SM and PAF, but the role of metal ions in catalysis or in the enzyme substrate interaction differs for the two substrates and requires further investigation. Table 1 is a comparison of the major features of alk-SMase activities against SM and PAF.

Table 1 Comparison of alk-SMase activities against SM and PAF

	SM	PAF
Cleavage	Phosphocholine	Phosphocholine
Optimal pH	9.0	7.5
TC, TCDC	Stimulating	Stimulating
Chaps	Inhibiting	Inhibiting
Triton X-100	Inhibiting	Inhibiting
EDTA	No effect	Inhibiting
Low concentration of Zn^{2+} (< 0.25 mM)	Inhibiting	Stimulating
High concentration of Zn^{2+} (> 0.25 mM)	Inhibiting	Inhibiting
Mutation of metal-binding site	Inhibiting	Inhibiting

The activity of alk-SMase against PAF was approx. 10 % of its activity against SM. There is a mutual inhibition in the sense that one substrate inhibited the activity against the other. The inhibitory effect of SM on PAF hydrolysis was stronger than that of PAF against SM hydrolysis when determined under the optimal conditions. The results indicate that the affinity and the catalytic capacity of the enzyme are higher for SM than for PAF. The physiological relevance of the results is not easy to assess, since the concentrations of SM in ileum and colon under physiological and pathological conditions are currently unknown. In the intestinal mucosa, alk-SMase activity against SM is approx. $10\ nmol \cdot h^{-1} \cdot (mg\ of\ mucosal\ protein)^{-1}$, and in faeces, it is approx. $0.5–1.0\ mmol \cdot h^{-1} \cdot (g\ of\ dry\ faeces)^{-1}$ [32]. The concentration of PAF in the intestinal tissue or intestinal lumen is low under normal conditions, approx. 4.5 ± 0.8 pg/mg of mucosal protein. The levels can be significantly increased under the inflammatory conditions. In stools of patients with Crohn's disease and ulcerative colitis, the PAF concentrations are 319.2 ± 143.5 and 824.9 ± 408 pg/g of stool ($\sim$0.5–1.4 pmol/g of stool) respectively [12]. Considering the high level of the enzyme in the middle and lower small intestine where bile salt concentrations should also be favourable for the activity of the enzyme, we postulate that the enzyme could hydrolyse substantial amounts of PAF located in this part of the gut.

Alk-SMase is expressed in the intestinal mucosa and released into the intestinal lumen by pancreatic trypsin [13,15]. PAF is present in the intestinal lumen where it is released from epithelial cells [33]. A large body of evidence indicates that PAF in the intestine, particularly in the colon, has several pro-inflammatory functions. PAF has been shown to have strong chemotactic effects [34], to enhance lymphocyte infiltration [35], to induce Cl^- secretion [9,36] and to cause diarrhoea [5]. It is the major factor responsible for endotoxin-induced diarrhoea [5,37] and necrotizing enterocolitis in the newborn [38]. PAF probably promotes inflammation via multiple mechanisms, including production and release of pro-inflammatory cytokines such as IL-8, TNFα and IL-1β [9,10,39], and activation of MAPK and NF-κB [8,40]. The increased cytokines may in turn stimulate the release of PAF [41], creating a positive feedback loop in accelerating the inflammatory response. Long-standing inflammation is a risk for carcinogenesis and PAF may play a role in colonic carcinogenesis, as its levels are increased in the inflamed tissues of ulcerative colitis and Crohn's disease [12], and colorectal carcinoma of Dukes' A and B stages [13]. Apart from the pro-inflammatory effects, PAF has also been found to affect the function of oncogenes,

such as stimulating the expression and action of VEGF (vascular endothelial growth factor) [42]. Effort has been made to discover novel drugs to inactivate PAF and attention is mainly focused on the cleavage of the acetyl group at C-2 position or on the interference of PAF receptor. To our knowledge, no agent that inactivates PAF by cleaving phosphocholine linked to the C-3 position has been reported. Our study for the first time identifies such a physiological factor in the intestinal tract. The alk-SMase-induced inactivation of PAF was demonstrated by the effects on the release of cytokine IL-8, the activation of MAPK and the chemotactic effect induced by PAF. *In vivo* inactivation of PAF by alk-SMase may block the noxious positive feedback and inhibit the inflammatory response in a short-term and carcinogenesis in a long-term perspective. Lack of a sufficient alk-SMase activity may increase the susceptibility of the mucosa to the effects of PAF, resulting in development of inflammation and cancer. In agreement with this hypothesis, a significant reduction of alk-SMase has been reported in long-standing ulcerative colitis [21] and in human colorectal carcinomas [19,43].

Our finding may also have important implications in liver diseases, because PAF was found to be associated with some types of liver damage, including carbon tetrachloride-induced liver cirrhosis [44], endotoxin- and alcohol-induced hepatic apoptosis and necrosis [45], and liver metastasis of colorectal carcinoma [46]. Alk-SMase is expressed also in human liver [17], and high alk-SMase activity was found in human bile [47,48]. Based on the findings in the present study, a protective effect of alk-SMase through hydrolysis and inactivating PAF in the liver and bile can be predicted.

In conclusion, the present study found a novel function of alk-SMase of hydrolysing and inactivating PAF by a PLC activity. This finding indicates a potentially important role of intestinal alk-SMase in preventing IBD and carcinogenesis in the gut.

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