A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signalling

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Sphingomyelinases hydrolyse sphingomyelin to ceramide, a process involved in signal-transduction routes leading to apoptosis and various other cellular responses. In the present study, we investigated the sphingomyelinase content of caveolae, invaginated plasma-membrane microdomains that contain a variety of signalling molecules. These structures are highly enriched in sphingomyelin as well as in ceramide, which suggests that metabolism of these lipids might, to some extent, occur locally. By cell fractionation, we demonstrate that, in addition to a previously reported minute amount of acidic sphingomyelinase activity, a substantial amount of neutral sphingomyelinase activity resides in caveolae of human skin fibroblasts. This caveolar neutral sphingomyelinase activity was also detected in Niemann–Pick disease type A fibroblasts, which are completely devoid of functional acidic sphingomyelinase. Neutral (but not acidic)

sphingomyelinase activity was specifically inhibited by a peptide that corresponds to the scaffolding domain of caveolin, which suggests a direct molecular interaction between the two proteins. In addition, this finding implies a cytosolic orientation of the caveolar neutral sphingomyelinase. Interestingly, stimulation of fibroblasts with tumour necrosis factor α (TNF α) resulted in a partial shift of its p55 receptor to caveolin-enriched membrane fractions and the appearance of caveolin-sensitive neutral sphingomyelinase activity in the non-caveolar fractions. These results suggest that (part of) the presently identified caveolar neutral sphingomyelinase activity is involved in TNF α signalling.

Key words: caveolae, ceramide, fibroblast, Niemann–Pick disease, TNF.

INTRODUCTION

Caveolae are dynamic microdomains of the plasma membrane (PM) that are present in many mammalian cell types, including fibroblasts [1–4]. Morphologically, caveolae appear as ≈ 70 nm flask-shaped invaginations of the membrane and a characteristic striated coat is often present. This coat is composed of caveolin, a ≈ 22 kDa integral membrane protein that is specifically expressed in mature caveolae, since it is absent from other detergent-insoluble complexes such as rafts, pre-caveolae or (so-called) caveolae-like domains [5,6]. In addition to morphological criteria, caveolae can be distinguished from the surrounding PM by biochemical criteria such as enrichment in (glyco)sphingolipids, cholesterol and glycosylphosphatidylinositol-anchored proteins. With respect to their function, caveolae have been implicated in transcytosis, potocytosis, cholesterol transport and cellular calcium regulation [7,8]. Furthermore, with the discovery of the caveolar location of a wide variety of signalling molecules, including epidermal-growth-factor, platelet-derived growth-factor and insulin receptors, non-receptor tyrosine kinases (Fyn, Src), G-proteins, Ras, nitric oxide synthase, adenylate cyclase, phosphatidylinositol 3-kinase and several protein kinase C isoforms, it has become evident that these structures play an important role in cellular signal transduction [9,10]. It has therefore been proposed that caveolae might act as relay stations that compartmentalize signal-transduction routes and facilitate cross-talk events [2,11]. The caveolin-scaffolding domain (CSD;

caveolin-1_{82–101}) provides a mechanistic basis for this hypothesis. This cytoplasmic 20-amino acid region of caveolin is envisaged to interact directly with caveolae-associated signalling molecules, of which many indeed contain caveolin-binding motifs [6,12].

The sphingolipid ceramide and its metabolites sphingosine and sphingosine 1-phosphate have been recognized as important mediators of signal-transduction processes leading to a variety of cellular responses, including apoptosis [13–15]. The principal metabolic source of these bioactive lipids is sphingomyelin (SM), a phospholipid that is ubiquitously present in the PM of mammalian cells. Sphingomyelinases (SMases; ceramide-phosphocholine phosphodiesterases, E.C. 3.1.4.12) are responsible for the hydrolysis of SM, and thus for the generation of ceramide. To date, at least seven different SMases have been distinguished, each differing in ion-dependency, pH optimum and subcellular location [16]. Although many details are known concerning the ‘housekeeping’ metabolism of SM, both the identity and the location of the putative signalling SMase(s) remain a source of intense debate [16,17]. Whereas several reports indicate the involvement of a lysosomal acidic SMase (aSMase) in signal transduction [18–20], other studies demonstrate a role for a PM-bound Mg²⁺-dependent neutral SMase (nSMase) in this respect [21–23]. New input into this discussion was provided by two reports on caveolae-associated ceramide generation upon stimulation of cells by interleukin-1 β or neurotrophins, respectively [24,25]. The first of these reports described the involvement of an aSMase in caveolar signalling.

Abbreviations used: SMase, sphingomyelinase; aSMase, acidic SMase; nSMase, neutral SMase; β -CDX, 2-hydroxypropyl- β -cyclodextrin; CSD, caveolin-scaffolding domain; NPD, Niemann–Pick disease; PC, phosphatidylcholine; PM, plasma membrane; SM, sphingomyelin; TNF α , tumour necrosis factor α .

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However, since both the cytosolic and extracellular faces of caveolae are principally neutral by pH, this finding might be considered as surprising. The second report provided no data on the nature of the involved SMase.

Bacterial SMase is able to induce microdomain formation in SM-containing model membrane systems [26]. Indeed, a local increase in ceramide can have a profound effect on the physical properties of a bilayer, for example on its curvature [26,27]. The typical flask-shaped curvature of caveolae is determined primarily by self-association of caveolin molecules [28], but local regulation of caveolar ceramide levels might be supportive in this respect. Therefore, in addition to a possible role in signal transduction, caveolar SMase activity might be involved in the formation and maintenance of microdomains. In this context the involvement of a nSMase has been suggested [27]. To date, however, no such caveolae-associated nSMase activity has been reported.

The issues described above led us to investigate the SMase content of caveolae further. Density-gradient fractionation of human skin fibroblasts resulted in the co-isolation of nSMase activity with caveolin. This nSMase, but not aSMase, was inhibited by a peptide that corresponds to the CSD, which suggests a direct molecular interaction between the enzyme and caveolin. Furthermore we obtained data that indicate a possible role of this nSMase–CSD interaction in tumour necrosis factor α (TNF α) signalling. These novel findings might have important implications for our understanding of ceramide-mediated signal-transduction processes, caveolae formation and, in general, the subcellular location of sphingolipid metabolism.

EXPERIMENTAL

Materials

Human skin fibroblasts were derived from control individuals and from patients with Niemann–Pick disease (NPD) type A. Cell-culture plasticware was from Nunc (Roskilde, Denmark), culture media and antibiotics were from Gibco-BRL (Paisley, U.K.) and foetal calf serum was purchased from Eurobio (Les Ulis, France). Rabbit polyclonal anti-(human caveolin) antibody was from Transduction Laboratories (Lexington, KY, U.S.A.). Goat polyclonal anti-(human CD71) antibody and rabbit polyclonal anti-(human TNF-R1) (TNF receptor 1 or TNF p55 receptor) were from Santa Cruz Biotechnology-Tebu (Le Perray-en-Yvelines, France). Hybond-C extra nitrocellulose blotting membrane, horseradish peroxidase-conjugated donkey anti-rabbit antibody and chemiluminescence detection reagents were from Amersham Pharmacia Biotech (Saclay, France). Protease inhibitors, 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide, 4-methylumbelliferone and 2-hydroxypropyl- β -cyclodextrin (β -CDX) were from Sigma (St. Louis, MO, U.S.A.). [*methyl*- 3 H]Choline (83 Ci/mmol), L-[3 H]serine (40 Ci/mmol) and [*methylcholine*- 14 C]SM (52 mCi/mmol) were from DuPont-NEN (Boston, MA, U.S.A.). The total cholesterol kit was purchased from Roche Diagnostics (Meylan, France). Dr T. Ogita (Sankyo Co., Tokyo, Japan) kindly provided scyphostatin. CSD and control (caveolin-1_{62–81}) peptides were synthesized by Sigma-Genosys (Cambridge, U.K.). TNF α was from R&D Systems (Minneapolis, MN, U.S.A.).

Cell culture

Both normal and NPD fibroblasts were immortalized by transfection with a plasmid encoding the simian virus 40 large T antigen [29]. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (4.5 mg/ml glucose) and U937 cells in RPMI

1640. Media were supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml) and 10% (v/v) heat-inactivated foetal calf serum. Cells were maintained in a water-saturated atmosphere of 5% CO₂/95% air at 37 °C. Stimulation with TNF α took place under low-serum conditions (1%).

Isolation of caveolae-enriched membrane domains

Caveolae were isolated from cells as described in [30]. For each isolation, confluent fibroblasts from a 175 cm² flask were washed twice with PBS and harvested by scraping. After pelleting by centrifugation, cells were resuspended in 1 ml of ice-cold Mes-buffered saline (MBS; 150 mM NaCl/25 mM Mes, pH 6.5) containing 1% (w/v) Triton X-100. After 30 min on ice, cells were homogenized further by 10 strokes of a tight-fitting Dounce homogenizer on ice. Ice-cold MBS (1.5 ml) was added and 2 ml of this suspension was mixed with 2 ml of 80% (w/v) sucrose in MBS. This mixture was subsequently loaded under a linear gradient consisting of 8 ml of 5–30% (w/v) sucrose in MBS. All solutions contained the following protease inhibitors: 100 μ M PMSF, 1 mM EDTA and 1 μ M each of aprotinin, leupeptin and pepstatin A. Gradients were centrifuged in a Beckman SW41 swinging rotor at 200 000 *g* for 20 h at 4 °C. Twelve fractions of 1 ml each were collected (from top to bottom), vortexed and stored at –80 °C. The protein content of both the fractions and the total initial cell suspension was measured using BSA as standard.

Immunoblotting

Samples were mixed with an equal volume of buffer that consisted of 2% (w/v) SDS, 20% (w/v) glycerol, 0.025% (w/v) Bromophenol Blue and 5% (v/v) β -mercaptoethanol in 125 mM Tris/HCl, pH 6.8. After heating for 4 min at 95 °C, aliquots of 20 μ l were subjected to SDS/PAGE and proteins were then electrotransferred on to blotting membrane. After blocking for 1 h with 10% (w/v) non-fat dried milk, membranes were incubated for 2 h with either rabbit polyclonal anti-(human caveolin), goat polyclonal anti-(human CD71) or rabbit polyclonal anti-(human TNF-R1) antibody. Subsequently, the membrane was washed and incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing, the membrane was incubated in chemiluminescence substrate solution, according to the manufacturer's instructions, and immunoreactive complexes were visualized by exposure of Kodak Biomax film.

β -Hexosaminidase activity

Each fraction (50 μ l) was mixed with 50 μ l of 0.2% (w/v) Triton X-100. Reactions were started by the addition of 50 μ l of substrate solution consisting of 2.6 mM 4-methylumbelliferyl *N*-acetyl- β -D-glucosaminide in 330 mM citrate/phosphate buffer, pH 4.8. After 10 min at 37 °C, the reactions were terminated by the addition of 2 ml of 200 mM ice-cold glycine, pH 10.5. The formation of reaction product (4-methylumbelliferone) was measured by spectrofluorometry at $\lambda_{\text{ex}} = 358$ nm and $\lambda_{\text{em}} = 448$ nm. Quantification took place with the aid of 4-methylumbelliferone standards.

Lipid analysis

Cellular pools of SM and phosphatidylcholine (PC) were metabolically radiolabelled by culturing cells for at least 48 h in the presence of 0.5 μ Ci/ml [*methyl*- 3 H]choline. Subsequently, cells

were washed and fractionated as described above. Lipids were extracted from 500 μ l of each fraction. In order to remove any remnants of free radioactive choline, the organic phases were washed with synthetic upper phase, consisting of chloroform/methanol/water (3:48:47, by vol.). Lipids were dried under N_2 and analysed as described in [17,31]. Briefly, the extracts were subjected to mild alkaline hydrolysis by overnight exposure of the lipids to 0.1 M NaOH in chloroform/methanol (1:1, v/v) at 37 °C. Lipids were re-extracted and the upper phases were collected. The lower phases were washed twice with synthetic upper phase. The latter two (washing) upper phases were pooled with the first and subjected to scintillation counting. This radioactivity represents the amount of PC that was present in the original extract. The remaining lower phase, representing the original pool of SM, was also subjected to scintillation counting. For radiolabelling of ceramide and glucosylceramide, cells were incubated with 3 μ Ci/ml L-[3 H]serine for at least 48 h. Free label was then washed away and cells were fractionated. Lipids were extracted from aliquots of 500 μ l and the extracts were analysed by TLC, using chloroform/methanol/32% (w/v) ammonia/water (70:25:3:1, by vol.) as the mobile phase. Radiolabelled lipids were detected by radiochromatoscanning and identification took place with the aid of standards.

Alternatively to the L-[3 H]serine labelling procedure, the endogenous ceramide contents of the fractions was determined using the diacylglycerol kinase assay [32]. In the case of TNF α -stimulation experiments, cells were allowed to stabilize for 3 h after washing of the cells. The total cholesterol contents of cell homogenates and fractions were determined automatically with the aid of a cholesterol oxidase-based commercial kit in combination with a Roche Cobas Mira analyser.

SMase activities

Aliquots of each fraction were assayed for the presence of SMase activity [22]. Volumes were adjusted to 250 μ l and adding 250 μ l of substrate solution started the reactions. For the measurement of aSMase activity, the substrate solution consisted of [methylcholine- 14 C]SM (1×10^5 d.p.m./assay) and 0.1% (w/v) Triton X-100 in 200 mM sodium acetate buffer (pH 5.0). In some cases this buffer was supplemented with 0.2 mM ZnCl $_2$. For nSMase assays the substrate solution consisted of [methylcholine- 14 C]SM (1×10^5 d.p.m./assay) and 0.1% (w/v) Triton X-100 in 200 mM Tris/HCl buffer (pH 7.4) containing 10 mM dithiothreitol and 10 mM MgCl $_2$. In some cases MgCl $_2$ was replaced by 10 mM EDTA. After 2 h of incubation at 37 °C, reactions were terminated by extracting the lipids. The amount of released radioactive phosphocholine was determined by subjecting an aliquot of the upper phase to scintillation counting. The amount of radiolabelled substrate that was hydrolysed during an assay never exceeded 10% of the total amount of substrate added.

Statistical analysis

For statistical analysis the unpaired two-way Student's *t* test was used. However, in the case of multiple comparisons the Tukey test was performed, after ANOVA.

RESULTS

Isolation and characterization of caveolae-enriched microdomains

The present study was performed on human skin fibroblasts, cells that are known to have a high caveolae content [3,33]. Caveolae-containing membrane domains were isolated from these cells according to a standard procedure [30]. This method takes advantage of the fact that caveolae are relatively resistant

Figure 1 Isolation and characterization of membrane microdomains

Cultured human skin fibroblasts were homogenized and fractionated by sucrose-density-gradient centrifugation as described in the Experimental section. The low-density fraction 1 was derived from the top of the gradient and the high-density fraction 12 from the bottom. Samples of each fraction were analysed for the presence of several marker proteins (left-hand panels); total protein (A), caveolin (B), CD71 (transferrin receptor, C) and β -hexosaminidase (D) activity. In addition, lipids were extracted from each fraction (right-hand panels) and analysed for SM (E), PC (F), ceramide (G), glucosylceramide (H) and cholesterol (I) contents. Data in the graphs are presented as the percentage (means $\pm$ S.D., $n = 4-11$ independent experiments) of protein, enzyme activity or lipid that was present in a fraction, as compared with the total amount on a gradient.

to detergent solubilization and have a low density, as compared with other cell organelles. Therefore, Triton X-100 extraction at 4 °C followed by density-gradient ultracentrifugation allows separation of caveolae-containing membranes from most, if not all, other organelles and membrane fractions [34].

After each ultracentrifugation run, an opaque band of insoluble material was consistently visible approximately halfway along the gradient. Immunoblotting against caveolin, the single most important marker protein for caveolae [6], revealed that the majority of this protein was present in the band-containing fractions (fractions 5–7, Figure 1B). Since this region of the gradient contained only a minor part of the total cellular protein content (4.1%, Figure 1A), caveolin must have been highly enriched in these fractions. The small quantity of caveolin that was detected in the denser part of the gradient (fractions 9–12)

Figure 2 SMase activities in microdomain-enriched membrane fractions

Fibroblasts were fractionated as described in the text, and samples of each fraction were assayed for the presence of SMase activities. nSMase activity was measured at pH 7.5 in the presence of MgCl_2 and dithiothreitol (A). aSMase was measured at pH 5.0 in the presence of EDTA (B). Enzyme activities present in each fraction are expressed as percentages of the total activity present on the gradient. Shown are means $\pm$ S.E.M. ($n = 7$ –16 independent experiments).

presumably originated from trans-Golgi-network fragments and/or transport vesicles. Figures 1(C) and 1(D) indicate that both CD71 (transferrin receptor) and β -hexosaminidase were present exclusively in fractions 9–12. Being marker molecules for non-caveolar PM and lysosomes respectively, this excluded a possible contamination of the caveolae-containing fractions by these organelles. Furthermore, the activity of the Golgi-specific enzyme ceramide: UDP-glucose transferase (glucosylceramide synthase) was present exclusively in fractions 9–12 (results not shown), a finding which thus rules out any Golgi contamination of the caveolae-containing fractions.

In addition to protein and enzymic measurements, the lipid composition of each fraction was analysed. Prolonged incubation of intact cells with [*methyl*- ^3H]choline resulted in metabolic radiolabelling of the cellular pools of both SM and PC. Upon fractionation of the cells and analysis of lipid extracts by TLC, 49.9% of all cellular SM then turned out to be present in fractions 5–7 (Figure 1E). In contrast, only 16.5% of the radiolabelled pool of PC was detected in these fractions. The majority of this lipid, which is an important constituent of non-caveolar PM [35], ended up in fractions 8–12 (Figure 1F). The distribution of ceramide over the gradients was studied by metabolic radiolabelling with L-[^3H]serine and subsequent TLC analysis. With 46.8% of the ceramide-incorporated radioactivity present in fractions 5–7, the distribution of ceramide over the gradients was quite comparable with that of SM (Figure 1G). Alternatively to radiolabelling, ceramide was measured by employing the diacylglycerol kinase assay [32], which confirmed the

observed enrichment in fractions 5–7 (results not shown). [^3H]Serine-labelled glucosylceramide also turned out to be highly enriched in the detergent-insoluble fractions (54.9% of the total in fractions 5–7, Figure 1H). Finally, a large quantity of cholesterol was detected in these fractions (52.5% of the total cellular cholesterol mass, Figure 1I).

In summary, application of the present procedure resulted in the purification of membrane domains that were highly enriched in caveolin, SM, ceramide, glucosylceramide and cholesterol. In contrast, non-caveolar PM markers (CD71 and PC), a Golgi marker (glucosylceramide synthase) and a lysosomal marker (β -hexosaminidase) were virtually absent from these fractions. This characterization is highly consistent with the existing literature on the molecular composition of caveolae. Furthermore, it has been reported that application of this procedure results in the exclusion of organelles such as nuclei, the endoplasmic reticulum and mitochondria from the caveolin-enriched fractions [34]. Taken together, we defined fractions 5–7 as the caveolae-containing fractions.

Microdomain-associated SMase activity

To gain insight into the SMase content of caveolae, we fractionated human skin fibroblasts as described above. The SMase assay, which is based on detection of the rate by which a radiolabelled headgroup is released from SM, distinguishes between the acidic and neutral activities by measurements at different pH values (5.0 and 7.5). In addition, the assays were performed in either the presence or absence of dithiothreitol, an inhibitor of aSMase, which has no effect on nSMases [36].

As shown in Figure 2(A), we detected a substantial amount (21.5% of the total cellular activity) of nSMase activity in the detergent-insoluble fractions. By performing the SMase assay in the presence or absence of 5 mM EDTA, we determined that the majority of this activity was Mg^{2+} -dependent, although the presence of a Mg^{2+} -independent form of nSMase could not be excluded (results not shown). In contrast to nSMase activity, virtually all cellular aSMase appeared to be present in the high-density bottom fractions of the gradient (Figure 2B). Since this distribution matches that of the lysosomal marker β -hexosaminidase (Figure 1D), this activity most probably represents the lysosomal pool of aSMase. However, detailed analysis revealed a minor peak (0.8% of total) of activity in fractions 5–7 (Figure 2B, insert). Since these particular fractions are devoid of any β -hexosaminidase activity (Figure 1D), lysosomal contamination can be excluded. When these measurements were repeated in the presence of ZnCl_2 , no increase in activity was observed (results not shown). Therefore, this minor peak may represent a non-lysosomal, and possibly caveolae-associated, pool of Zn^{2+} -independent aSMase activity.

In most cell types, aSMase activity is much more abundant than nSMase activity. With respective absolute specific activities of 2651 ± 885 ($n = 11$) versus 62 ± 23 ($n = 16$) pmol $\cdot$ mg of protein $\cdot$ h $^{-1}$ in total cell lysates, this is clearly also the case for human skin fibroblasts. The amount of aSMase activity that co-fractionated with caveolin is relatively small. In terms of absolute specific activity, however, this might well be comparable with the amount of caveolae-associated nSMase. Despite the presence of dithiothreitol and despite the fact that nSMase assays were performed at pH 7.5, the possibility therefore remained that the observed caveolae-associated nSMase activity might in fact be attributed to some residual aSMase activity. We therefore performed a control experiment on fibroblasts that were derived from a NPD type A patient. Due to an inherited defect in the

Figure 3 nSMase activity in microdomains of aSMase-deficient fibroblasts

Total homogenates of human skin fibroblasts that were derived from a NPD type A patient were assayed for aSMase activity and compared with normal fibroblast homogenates (A). nSMase activity was measured in fractionated NPD cells (B). The enzyme activity present in each fraction is expressed as a percentage of the total activity on the gradient. Shown are means $\pm$ S.E.M. ($n = 5$ independent experiments). The insert shows an immunoblot against caveolin. For this experiment equal amounts (5 μ g) of total homogenates of normal and NPD fibroblasts were resolved by SDS/PAGE and immunoblotted with anti-caveolin.

aSMase gene, this enzymic activity is virtually absent from these cells (Figure 3A) [36]. When NPD fibroblasts were fractionated and tested for nSMase activity, a distribution pattern was found that was comparable with that of control fibroblasts (Figure 3B). The relative amount of caveolae-associated nSMase activity appeared to be slightly lower than in normal fibroblasts (15.4% versus 21.5%). Interestingly, NPD cells also expressed less caveolin when compared with control cells (Figure 3B, insert).

An additional control experiment was performed by employing scyphostatin, a recently identified nSMase inhibitor that was derived from *Dasyatis mollissima* [37]. The specificity of this compound was demonstrated by the observation that nSMase activity in total cell homogenates was substantially inhibited, whereas aSMase remained totally unaffected (Table 1). In particular the Mg^{2+} -dependent form of nSMase was sensitive to this inhibitor, whereas the Mg^{2+} -independent form was relatively insensitive (results not shown). nSMase activity in caveolin-enriched membranes (pooled fractions 5–7 of a gradient) was significantly inhibited when scyphostatin was included in the reaction mixture, whereas aSMase again remained unaffected (Table 1). This finding confirms that the observed caveolae-associated SMase activity is truly neutral by nature.

Application of the present isolation procedure results in the co-purification of caveolae with other types of membrane microdomain, if present. In order to determine whether the observed

Table 1 Inhibition of nSMase by scyphostatin

Cells were fractionated as described and both caveolin-enriched fractions (5–7) and high-density fractions (9–11) were pooled and assayed for SMase activities in the presence of 20 μ M scyphostatin, or in its absence (controls; results not shown). Activities were also measured in total cell homogenates. Data are expressed as percentages (means $\pm$ S.D., $n = 4$ –7) of the untreated controls, which were set at 100%. Asterisks indicate significant differences from the untreated controls ($P < 0.01$). n.d., not determined.

	Fractions 5–7	Fractions 9–11	Total homogenates
nSMase	54.9 $\pm$ 11.9*	64.6 $\pm$ 12.8*	54.3 $\pm$ 8.2*
aSMase	100.7 $\pm$ 9.1	n.d.	100.2 $\pm$ 7.0

Figure 4 nSMase is present in caveolin-free microdomains of U937 cells

U937 myeloid cells were fractionated and analysed for the presence of nSMase. Data are expressed as the percentage of enzyme activity in a fraction, as compared with the total activity that was present on a gradient (means $\pm$ S.E.M., $n = 7$ independent experiments). The insert shows an immunoblot against caveolin. For this experiment, 5 μ g of total U937 cell homogenate was resolved by SDS/PAGE and immunoblotted with anti-caveolin (right-hand lane). Fibroblast homogenate (5 μ g) served as a positive control (left-hand lane).

nSMase activity is specific for caveolae or, alternatively, a more general feature of microdomains, we repeated the nSMase measurements on fractionated U937 cells (Figure 4). These myeloid cells are completely devoid of caveolin (Figure 4, insert), and thus of caveolae [38]. Nevertheless, some nSMase activity was detected in the detergent-insoluble fractions of these cells (10.8% of the total cellular activity). Therefore, nSMase activity in microdomains does not appear to be an exclusive feature of caveolae.

Cholesterol depletion induces redistribution and activation of nSMase

Local cholesterol levels are an important factor with respect to the structure and function of caveolae [39]. Indeed, treatment of intact cells with cholesterol-depleting agents such as β -CDX has been reported to cause a loss of caveolar integrity and to result in a redistribution of caveolae-associated molecules [40]. Human fibroblast microdomains are enriched in cholesterol (Figure 1I). Treatment of intact cells for 1 h with 10 mM β -CDX (before fractionation) resulted in an overall decrease in cholesterol mass to 45.2 $\pm$ 8.9% compared with untreated control cells (Figure 5A). Concomitantly, the total cellular nSMase activity increased

Figure 5 Cholesterol removal activates nSMase

Confluent cultures of fibroblasts were treated for 1 h with 10 mM β -CDX. After washing, cells were homogenized and total cholesterol was measured (**A**). Data are expressed in nmol/mg of cellular protein as means $\pm$ S.E.M. ($n =$ four independent experiments). (**B**) The activities of nSMase in fractionated control (○) or β -CDX-treated (●) cells are shown. Enzyme activities are expressed as pmol·mg of protein·h⁻¹ of total protein on the gradient. Shown are means $\pm$ S.E.M. ($n =$ three independent experiments).

Figure 6 Specific inhibition of nSMase by the CSD

Total homogenates of fibroblasts were assayed for neutral (**A**) or acidic (**B**) SMase activity as described in the Experimental section. The assays were performed in the presence of the indicated concentrations of a 20-amino acid peptide representing the CSD (caveolin-1₈₂₋₁₀₁; ●). Alternatively, a 20-amino acid control peptide was used (caveolin-1₆₂₋₈₁; ○). Enzyme activities are expressed as mean percentages $\pm$ S.E.M., as compared with the activities in the absence of peptide ($n = 3-4$ independent experiments). Asterisks indicate a significant difference ($P < 0.05$) between the inhibiting effect of the CSD and the effect of the same concentration of control peptide.

to 115.1%. Analysis of fractionated cells revealed that this additional nSMase activity appeared exclusively in the non-caveolar high-density part of the gradient (Figure 5B). This

Figure 7 TNF α activates a CSD-inhibitable pool of nSMase

Normal human skin fibroblasts were stimulated for 30 min by 25 ng/ml TNF α or control vehicle under low-serum conditions (1% foetal calf serum). Subsequently, cells were washed and fractionated. (**A**) Western blots against the 55 kDa TNF α receptor R1 are shown. TNF α effects on lipid levels were studied after lipid pre-labelling by incubating the cells for 48 h with radiolabelled precursor molecules (**B**). Changes in SM (▲) and PC (○) pools were monitored by radiolabelling with [*methyl*-³H]choline, whereas ceramide was studied after L-[³H]serine pre-labelling (●). Data are expressed as the percentage of change of a lipid in a particular fraction, as compared with the corresponding fraction derived from untreated control cells (means $\pm$ S.E.M., $n = 3-5$ independent experiments). In addition to the lipid levels, changes in nSMase activity were studied directly (**C**). For this, caveolin-enriched fractions (5-7) were pooled, as were fractions derived from the high-density region of the gradient (9-11). Samples were then assessed for nSMase activity in the presence or absence of 40 μ M CSD peptide (cav-1₈₂₋₁₀₁). Activities are expressed as pmol·mg of total protein·h⁻¹ $\pm$ S.D. ($n = 3-6$). The different experimental conditions (control or TNF α pre-treatment of the cells and the presence or absence of the CSD peptide during the nSMase assay) are indicated by symbols underneath the panels. All differences between treatments mentioned in the text are significantly different from each other ($P < 0.05$), calculated as described in the Experimental section.

finding suggests that cholesterol removal from microdomains such as caveolae induces a release of nSMase activity into the detergent-soluble parts of the cell, most likely the cytosol or the non-caveolar PM.

The CSD specifically inhibits nSMase

The scaffolding domain of caveolin inhibits various signalling molecules [12]. We investigated whether the presently identified microdomain-associated nSMase activity is similarly regulated

by the CSD. When nSMase activity of total cell lysates was measured in the presence of a peptide corresponding to the CSD, a significant dose-dependent inhibition of the enzyme was observed, starting at 10 μ M (Figure 6A). It should be noted that at concentrations as high as 40 μ M, the CSD only partially inhibited nSMase. At even higher concentrations, the level of inhibition never exceeded 50 % of the total cellular activity (results not shown). The experiments were repeated with a control peptide of equal length, but representing another region of caveolin (caveolin-1₆₂₋₈₁). In this case, no inhibition of nSMase was observed (Figure 6A). In contrast to nSMase, aSMase turned out to be completely insensitive to both the CSD and the control peptide (Figure 6B). Taken together, the observed inhibition appears to be specific with respect to both the region of caveolin and the involved SMase.

TNF α stimulation increases nSMase activity in non-caveolar compartments

To investigate the possible involvement of the newly identified caveolar nSMase in signal-transduction processes, we stimulated intact fibroblasts for 30 min with 25 ng/ml TNF α , a well-known physiological activator of the SM-ceramide pathway [13–16]. As demonstrated by Western blotting, this treatment resulted in a partial relocation of the TNF α p55 receptor from the detergent-soluble to the detergent-insoluble fractions (Figure 7A). Concomitantly, TNF α activated the SM-ceramide pathway. In total cell homogenates a decrease in SM levels to 86.2 ± 11.1 % was observed, whereas ceramide levels increased to 126.9 ± 7.4 % (both compared with control-treated cells). However, these changes were not observed in caveolin-enriched fractions, but rather in the high-density fractions (Figure 7B). In addition to the lipid levels, direct measurements of nSMase activity were performed on TNF α -stimulated (and subsequently fractionated) cells (Figure 7C). Although a small increase in microdomain-associated nSMase was detected, a significant increase was only observed in non-caveolar, high-density fractions.

As described above the CSD partially inhibited nSMase activity in total cell homogenates (Figure 6). We analysed the subcellular distribution of this CSD-sensitive nSMase by measuring nSMase activities in fractions in the presence of 40 μ M CSD peptide. Surprisingly, the CSD exclusively inhibited nSMase that was present in the caveolin-enriched fractions (36.3 % as compared with control values, Figure 7C, left-hand panel). nSMase that resided in the non-caveolar fractions was not sensitive to the CSD (97.6 % activity in the presence of 40 μ M CSD peptide). Interestingly, however, when cells were stimulated beforehand with TNF α , the nSMase that appeared in the high-density region of the gradient became sensitive to inhibition by the CSD peptide (Figure 7C, right-hand panel).

DISCUSSION

The molecular composition of detergent-insoluble membrane microdomains such as caveolae differs greatly from that of the surrounding PM and other subcellular membranes. Of principal interest for the present study is the caveolar enrichment in SM. Catabolism of SM occurs mainly by the action of an aSMase during membrane turnover in the endosomal/lysosomal pathway [15]. However, the fact that caveolae are also highly enriched in ceramide suggests that SM hydrolysis might, at least partly, occur locally. The present study provides evidence for the existence of caveolar, mainly Mg²⁺-dependent, nSMase activity.

This enzymic activity can be inhibited by scyphostatin, a specific nSMase inhibitor [37]. Microdomain-associated nSMase activity is also present in cells that lack functional aSMase (NPD type A cells). Interestingly, NPD cells express substantially less caveolin than normal fibroblasts, a finding that is in accordance with a recent report which describes alterations in microdomains in cells that were obtained from aSMase-deficient mice [39]. In addition to nSMase, a small but detectable quantity of Zn²⁺-independent aSMase activity was detected, which is in agreement with previous reports [24,25].

Several different nSMases have so far been identified in mammalian cells, of which some have recently been purified [41,42] and/or cloned [43], among which are nSMase1 [44] and nSMase2 [45]. Fractionation of fibroblasts that were transfected by a cDNA encoding for nSMase1 demonstrated that the additional enzymic activity was fully targeted to non-caveolar compartments of the cell. This finding was confirmed by a confocal microscopy study in which cells were examined that over-expressed a green fluorescent protein–nSMase1 hybrid protein. Labelling was present exclusively at intracellular sites and no co-localization with anti-caveolin immunostaining was observed (R. J. Veldman, unpublished work). These findings are in accordance with recent reports that describe nSMase1 to be located in the endoplasmic reticulum [46,47], whereas nSMase2 is present predominantly in the Golgi apparatus of brain cells [45]. Taken together, these data suggest that the presently identified caveolar nSMase activity is distinct from the enzymes encoded by the cloned cDNAs.

Antibodies against membrane-bound nSMases are not commercially available. Therefore, direct demonstration of the caveolar nSMase protein by means of Western blotting, immunohistochemistry, immunoelectron microscopy or immunoprecipitation is beyond present experimental possibilities. However, in addition to co-fractionation of nSMase with caveolin, we did obtain evidence that suggests a direct molecular interaction between the two proteins. Namely, the scaffolding domain of caveolin specifically inhibits the activity of nSMase. However, this inhibition never exceeded 50 % of the total activity in cell lysates, independent of the concentration of CSD peptide. This finding suggests that the total nSMase activity in fibroblasts might be divided into two pools; one that is sensitive to the CSD and another that is not. nSMase activity that is present in the detergent-insoluble fractions is more strongly inhibited by 40 μ M CSD peptide (36.3 %) than the overall cellular nSMase activity (56.9 %), and much more strongly than the activity from non-caveolar fractions (97.6 %). This indicates that the CSD-sensitive pool of nSMase is mainly restricted to caveolae. However, if caveolin inhibits nSMase activity *in vivo*, the question remains as to why any nSMase activity is detected at all in the caveolin-enriched fractions. Apart from a possible dissociation, and thus activation, of nSMase from caveolin during the isolation procedure, the possibility exists that part of the observed nSMase activity resides in rafts or other caveolin-free microdomains. Indeed we show that U937 cells, which do not contain caveolae, nevertheless do contain some nSMase activity in their detergent-insoluble fractions (Figure 4). With respect to fibroblasts, however, very little is known concerning the presence of rafts and their relative abundance, as compared with caveolae. The possibility therefore exists that the observed activity in the detergent-insoluble fractions can be accounted for by a raft-associated nSMase. A final possibility that could explain the observed nSMase activity in caveolin-enriched microdomains might be that the CSD does not completely block the enzyme, but rather acts as a modulator, which then results in some residual activity.

The CSD is present on the N-terminal, cytoplasmic part of caveolin [12]. The finding that caveolar nSMase is inhibited by the CSD, and not by other domains of caveolin, therefore indicates that this molecular interaction occurs at the cytoplasmic leaflet of the caveolar membrane. In contrast, we hypothesize that the caveolar pool of aSMase activity will be orientated towards the lumen of caveolae, e.g. the extracellular space. Namely, the presence of an enzyme with an acidic pH optimum in an organelle that is principally neutral by nature is rather unexpected. One requirement for optimal enzyme activation would be a temporary acidification of the organelle. This might, however, be envisaged since a caveolar proton ATPase has been demonstrated [48]. An obvious prerequisite for acidification would then be a transient physical isolation of the internal milieu from the neutral extracellular space. Since fibroblast caveolae have the ability to internalize, this might well be a key event in caveolar aSMase activation [49,50]. An important implication of this model would be an orientation of the aSMase (and hence ceramide generation) towards the lumen of the caveolae, e.g. the extracellular space. The present observation that aSMase is not inhibited by the cytoplasmic orientated CSD fits within this model.

The finding of both neutral and acidic SMases in caveolae, and possibly in other microdomains as well, raises questions concerning their function. In many cell types, including fibroblasts, triggering of receptors for interleukin-1 β , TNF α or CD95, or the application of non-specific stress stimuli such as ionizing radiation or serum deprivation, leads to the formation of bioactive ceramide [13–15]. The involvement of both nSMases and aSMase has been reported in this respect [16]. In close relation to the nature of the enzyme, the exact subcellular site of ceramide release is an important factor that may determine the eventual biological effect of the lipid. Two previous studies have described SMase activity in caveolae, and both implied SMase activity in signal transduction. The first claimed the involvement of an aSMase in the generation of caveolar ceramide upon stimulation by interleukin-1 β , although no cytokine-induced increase in enzyme activity was shown [24]. For reasons unclear to us, nSMase activity was not detected in their system, despite the fact that this study was also performed on human fibroblasts, using an identical caveolae isolation procedure. The second report described nerve growth factor-induced hydrolysis of caveolar SM, but provided no further information on the nature of the involved SMase [25]. With the present study, we demonstrate for the first time that in fact both forms of SMase are present in caveolae and/or other microdomains.

With respect to signal transduction, we focused our experiments on nSMase. Although indeed activated by TNF α , this enzymic activity was only observed in the non-caveolar parts of the cells, presumably the cytosol and/or non-caveolar PM. Concomitantly, SM hydrolysis and ceramide generation took place exclusively in these detergent-soluble fractions. An obvious explanation for this would be that TNF α activates a nSMase that is present in the cytosol or non-caveolar PM, thereby excluding a signalling role for any caveolar nSMase. However, as demonstrated by Western blotting, TNF α stimulation results in a partial relocation of TNF-R1, which suggests that some of the downstream signalling events occur in caveolae and/or other microdomains (Figure 7A). In addition, we observed that TNF α -activated nSMase is inhibitable by the CSD, whereas no such inhibition of non-caveolar nSMase(s) was ever observed under non-stimulated conditions (Figure 7C). These observations led us to a model in which caveolar nSMase is kept in an inactive state due to the specific inhibition by the CSD. However, TNF α stimulation induces a redistribution of its p55 receptor to

caveolae, which in turn results in a release, and thus activation, of the nSMase. Originating from the cytoplasmic side of caveolae, the enzyme now has access to the pool of SM that is present in the inner leaflet of the PM and which is generally believed to be involved in signal-transduction processes [31,51–53]. In accordance with this model is the observation that disruption of caveolae by cholesterol depletion results in a nSMase activation in non-caveolar compartments of the cell (Figure 6). Although direct activation of non-caveolar nSMase via non-caveolar TNF receptors cannot be excluded, nSMase originating from caveolae may account for (at least part of) the observed ceramide generation.

In both cases the question remains as to why no SM hydrolysis was observed in the caveolar fractions, despite the fact that these structures are highly enriched in SM [2,24]. Experiments in which SM pools from the outer leaflet of the PM (and thus of caveolae) were depleted by an exogenously applied bacterial SMase (which does not have access to intracellular pools), suggested that caveolar SM is virtually exclusively present in the outer leaflet of the membrane (R. J. Veldman and T. Levade, unpublished work). Therefore, this pool of SM is inaccessible to the TNF α -activated and cytoplasmic-oriented nSMase, independent of the exact mechanism involved.

As discussed above, the interaction of the CSD with the caveolar nSMase is inhibitory by nature. This is in close agreement with previous observations on many other caveolae-associated signalling molecules [6,12,54,55]. The interaction of caveolin with signalling molecules is presumed to occur through binding of the CSD to a specific motif, which has been identified in many signalling proteins [12]. Analysis of published amino acid sequences revealed that none of the presently cloned nSMases contains such a caveolin-binding motif, which is in accordance with both the non-caveolar location of these SMases [45,47] and their lack of signalling properties [44]. Cloning and analysis of other nSMases will reveal whether a caveolin-binding motif is present in one or more of these enzymes.

In addition to their possible implication in signalling, we envision a role for caveolar SMases in the formation of microdomains such as caveolae. Local increases in ceramide content can have profound effects on the physical properties of a bilayer [26,27], possibly due to ceramide–ceramide interactions by hydrogen bonding [56]. Direct SM–SM interactions occur less readily, probably due to steric hindrance by the hydration shell of the phosphocholine headgroup [26]. Interestingly, treatment of SM-enriched model membranes with a bacterial SMase results in the formation of ceramide-containing microdomains [26]. Furthermore, it has been hypothesized that the space provided by SM-headgroup removal is required for membrane-bending processes [27]. Self-association of caveolin molecules, and their interaction with lipid components such as cholesterol, is the principal driving force behind caveolae formation [28]. In this respect, however, we hypothesize that cellular SMases, which concomitantly decrease SM and increase ceramide levels, might facilitate microdomain formation and membrane bending. With the present study, we demonstrate that caveolae, and possibly other microdomain forms as well, do indeed contain SMase activities.

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