

# Localization of neutral ceramidase in caveolin-enriched light membranes of murine endothelial cells

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**Abstract** Sphingomyelinase (SMase) and ceramidase (CDase) activities participate in sphingomyelin (SM) metabolism and have a role in the signal transduction of a variety of ligands. In this study evidence is presented that caveolin-enriched light membranes (CELMs) of murine endothelial cells, characterized by high SM, ceramide (Cer) and cholesterol content, bear acid and neutral SMase as well as neutral CDase activities. Localization of neutral CDase in CELMs was confirmed by Western analysis. Notably, cell treatment with cyclodextrin, which depleted cell cholesterol, did not affect acid or neutral SMase activities but significantly enhanced neutral CDase activity in CELMs, indicating a negative role for cholesterol in CDase regulation. These findings suggest that neutral CDase is implicated, together with SMase activities, in the control of caveolar Cer content that may be critical for caveola dynamics. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** Neutral ceramidase; Caveolin-enriched light membrane; Cholesterol; Murine endothelial cell

## 1. Introduction

Extensive work performed over the last decade has highlighted the role of sphingolipids and their metabolites as bio-modulators of multiple cell functions [1,2]. Various ligands are known to elicit their biological responses through the signaling pathway generated by the sphingomyelinase (SMase)-catalyzed hydrolysis of sphingomyelin (SM), that generates ceramide (Cer) and phosphocholine [2,3]. Cer, backbone of all sphingolipids, has been shown to be involved in the regulation of multiple biological processes including cell differentiation and apoptosis [3,4]. Ceramidases (CDases) are enzymes that hydrolyze the *N*-acyl linkage of Cer into sphingosine (Sph) and free fatty acid. Current knowledge indicates that Sph is not produced by de novo synthesis [5], thus crucially implicating CDase not only in the turn-off of Cer-induced signaling

pathways but also in the formation of Sph, which can be phosphorylated to Sph 1-phosphate (S1P) by Sph kinase. Notably, S1P, which acts as extracellular ligand as well as an intracellular mediator, is recognized to exert powerful biological actions including mitogenic and antiapoptotic effects in a number of cell types [6]. Similarly to what is observed for SMase, multiple isoforms of CDase have been reported, mainly differing by their catalytic pH optima. Molecular cloning of neutral CDase recently performed in mouse [7], human [8] and rat [9] has shown the occurrence of at least three distinct membrane-bound isoforms, which may also differ for subcellular localization.

Caveolae are flask-shaped invaginations of the plasma membrane coated by a 22 kDa structural protein denominated caveolin (cav) [10], initially detected in endothelial cells and successively individuated in many cell types. Such microdomains are highly enriched in SM and cholesterol [11,12] and are distinguishable from bulk cellular membranes by their resistance to detergent solubilization [13]. Caveolae have been implicated in a wide range of cellular functions including transcytosis, receptor-mediated uptake, stabilization of lipid rafts and compartmentalization of a number of signaling events at the cell surface [11–13]. Remarkably, approximately half of cellular Cer appears to be located in sphingolipid-enriched domains [14,15] and, on the basis of profound changes in the biophysical properties of membranes induced by increases of Cer in membrane bilayers, an active function of Cer in raft organization and trafficking has been proposed [16]. Intriguingly, increase of Cer in caveolar microdomains was found to be elicited by interleukin 1 $\beta$  [14] and nerve growth factor [17] supporting the view that Cer content of caveolar microdomains could be regulated by extracellular signals. Consistent with these findings, acid SMase was detected in caveolae fractions of human fibroblasts [14] and very recently neutral and acid SMases were both identified in sphingolipid-enriched domains of the same cell type [18]. However, at present it is not known whether Cer localized in caveolae can be further metabolized to Sph in these microdomains. In view of the scarce propensity of Cer to interbilayer movements [19], this issue could be of importance in the comprehension of the mechanisms involved in the regulation of the lipid membrane content. In this study we present evidence that neutral CDase resides in cav-enriched light membranes (CELMs) of murine endothelial cells, together with acid and neutral SMase activities. Of note, exclusively neutral CDase activity resulted to be positively modulated upon cho-

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**Abbreviations:** SMase, sphingomyelinase; SM, sphingomyelin; Cer, ceramide; CDase, ceramidase; Sph, sphingosine; cav, caveolin; CELM, caveolin-enriched light membrane; IM, intermediate density membrane; HM, high density membrane; CD, cyclodextrin

lesterol depletion, suggesting that the regulation of enzyme specifically depends on membrane cholesterol levels.

## 2. Materials and methods

### 2.1. Materials

H.end.FB murine endothelial cells were a kind gift of Prof. F. Bussolino (University of Turin, Turin, Italy). [ $^3\text{H}$ ]Palmitic acid (30–60 Ci/mmol) and [ $^3\text{H}$ ]acetic acid (2–5 Ci/mmol) were purchased from Du Pont NEN (Boston, MA, USA). Reagents, materials for cell cultures, cyclodextrin (CD) were obtained from Sigma (St. Louis, MO, USA). BODIPY FL-SM and BODIPY FL-Cer were obtained from Molecular Probes (Leiden, The Netherlands). C12-NBD-Cer was prepared by the method described in [20]. Precoated silica gel 60 thin layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). I-block was from Tropix (Bedford, MA, USA). Neutral recombinant mouse CDase was purified and utilized to generate rabbit polyclonal antibodies as described in [9]. Rabbit polyclonal anti-calnexin antibodies were purchased from Stressgen Biotech (Victoria, BC, Canada). Mouse monoclonal antibodies against cav-1 and horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG1 were from Santa Cruz (Santa Cruz, CA, USA).

### 2.2. Cell culture

H.end.FB cells were grown in 100 mm dishes in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and penicillin G (100 U/ml) plus streptomycin (100  $\mu\text{g}/\text{ml}$ ), maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  and utilized for experiments when 90% confluent.

### 2.3. Purification of cav-enriched membranes

Cells were washed twice with ice-cold PBS, scraped into MES-buffered saline (MBS) (25 mM MES, pH 6.5, 0.15 M NaCl, 1% Triton X-100) containing a mixture of protease inhibitors and homogenized with a Dounce homogenizer (100 strokes). Lysates were subjected to sucrose gradient centrifugation essentially as described in [21] with minor modifications. The homogenate was adjusted to 40% sucrose by 1:1 addition of 80% sucrose prepared in MBS, placed at the bottom of an ultracentrifuge tube and overlaid with two layers of 30% and 5% sucrose in MBS. The gradient was then centrifuged at 170 000  $\times g$  for 18 h using a Beckman SW50 rotor. For analysis of the resulting gradient, 0.4 ml fractions were collected from the top of the gradient. Protein content was quantified according to the Coomassie blue procedure.

### 2.4. Cholesterol depletion

Cholesterol depletion was carried out according to [22] by incubating cells with 14 mM CD for 1 h at 37°C. In some experiments cells were replenished with cholesterol by incubating them in the presence of cholesterol/CD mixture (0.2 mM cholesterol and 14 mM CD) for 1 h.

### 2.5. Lipid analysis

For the determination of SM and Cer levels in sucrose density fractions, cellular pools were metabolically radiolabeled with [ $^3\text{H}$ ]palmitate (0.7  $\mu\text{Ci}/\text{ml}$ ) for 24 h in the presence of 10  $\mu\text{M}$  unlabeled palmitate [23]. Under these conditions, sphingolipids were labeled well, and palmitate was broken down to acetate and recycled such that de novo synthesized cholesterol was also labeled. Cell lysates were then subjected to sucrose gradient fractionation and lipids were extracted. SM was separated by TLC in a solvent system containing: chloroform/dimethylketone/methanol/acetic acid/water (60:24:18:12:6, v/v) [14]. Cer was separated by TLC using chloroform/methanol/ammonia/water (80:20:1:1, v/v) up to 9.5 cm and then with diethyl-ether/methanol (99:1, v/v) up to 19.5 cm [24].

To determine cholesterol distribution in sucrose density fractions, cells were metabolically radiolabeled with [ $^3\text{H}$ ]acetate (10  $\mu\text{Ci}/\text{ml}$ ) for 24 h, in the presence of 10  $\mu\text{M}$  unlabeled acetate [25]. Cell lysates were then subjected to sucrose gradient fractionation, lipids were extracted and separated by TLC in a solvent system containing benzene/ethyl-acetate (60:20, v/v) [26].

### 2.6. Enzymatic assays

CDase activity was determined using C12-NBD-Cer as a substrate [27]. Briefly, 100 pmol of C12-NBD-Cer (NBD-C12:0, d18:1) was incubated at 37°C for 2 h with an appropriate amount of protein in

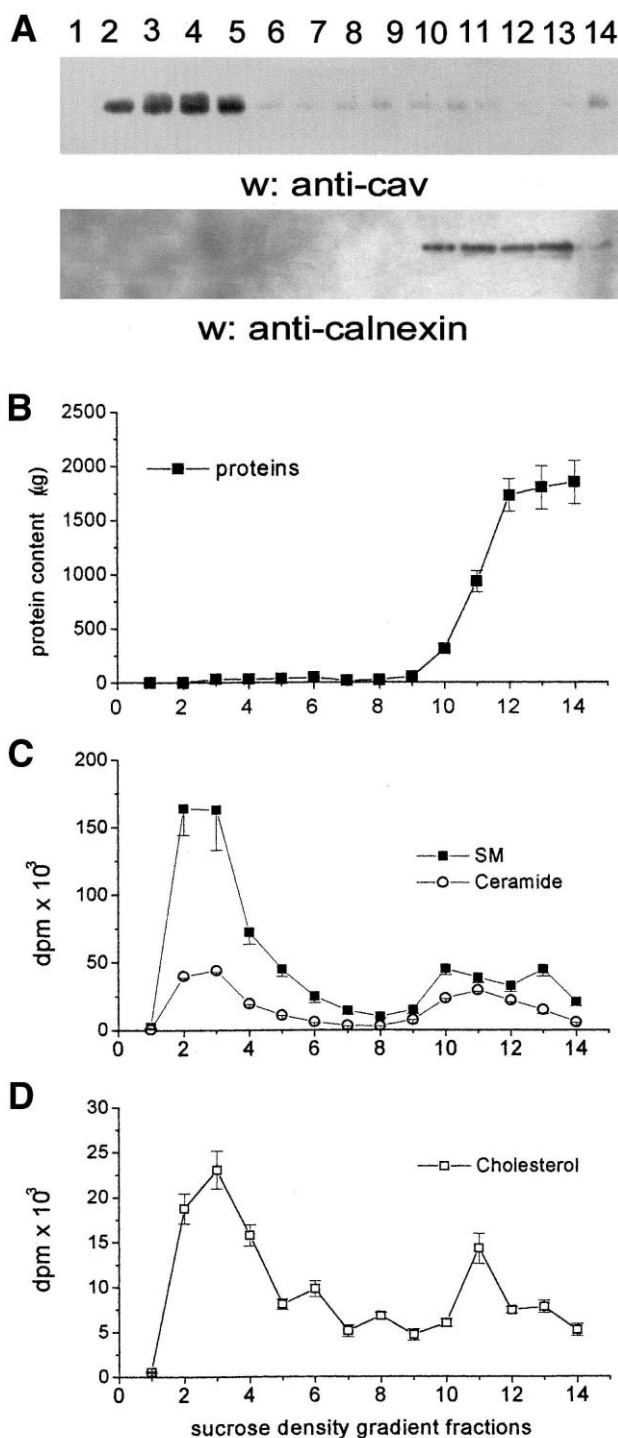


Fig. 1. Sucrose density gradient fractionation of H.end cells. H.end cell lysates were fractionated by sucrose density gradient centrifugation. Fractions of 0.4 ml were analyzed for marker proteins cav-1 or calnexin (A), total protein (B), SM and Cer (C) and cholesterol contents (D). Data of lipid distribution are means  $\pm$  S.E.M. of a representative experiment of at least three performed in triplicate. When not shown the error bar lies within the symbol.

20  $\mu\text{l}$  of 25 mM Tris-HCl buffer, pH 7.5 and 0.25% (w/v) Triton X-100. Samples were then applied to a TLC plate, which was developed with chloroform, methanol, 25% ammonia (90:20:0.5, v/v). Spots corresponding to NBD-dodecanoic acid and C12-NBD-Cer were scraped, incubated with methanol at 37°C to extract the compounds from the silica and their fluorescence at 470/525 nm excitation/emission wave-

lengths was measured. The compounds were quantified using a standard curve of known amounts of C12-NBD-Cer and NBD-dodecanoic acid.

For the measurement of acid and neutral SMase activities we utilized a spectrophotofluorimetric assay based on detection of the rate by which BODIPY FL-Cer was released from BODIPY FL-SM, essentially as previously described [28]. pH values of the enzyme assay mixture were adjusted to 5.0 and 7.4 for acid and neutral SMase, respectively.

### 2.7. Immunoblotting

Protein aliquots from sucrose density gradient fractions were separated by SDS-PAGE and then electrotransferred to nitrocellulose membranes, which were incubated overnight in Tris-buffered saline containing 0.1% Tween-20 (TTBS) and 0.5% I-block. Hybridization for 1 h at room temperature with primary antibodies was followed by washing with TTBS and incubation with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG1. Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

### 2.8. Statistical analysis

The data were analyzed by Student's *t*-test and  $P < 0.05$  was considered significant. In immunoblot experiments a blot representative of at least three similar experiments is presented.

## 3. Results

H.end cells are immortalized cells which maintain the *in vitro* features of endothelial cells [29]. In agreement with endothelial phenotype, they were recently shown to be characterized by high levels of cav [30]. To isolate CELMs, in this study, H.end cells were lysed in the presence of Triton X-100 and fractionated on a discontinuous sucrose density gradient. The obtained fractions (0.4 ml) were analyzed for cav immunoreactivity. As shown in Fig. 1A, cav was enriched in fractions 2–5 (CELMs), which corresponded to the turbid interphasic band between 30 and 5% sucrose equilibrium density. CELMs excluded the intracellular membrane protein calnexin which was instead enriched in fractions 10–13. Analysis of protein profile demonstrated that the bulk of protein was present in the high density sucrose fractions (Fig. 1B). Typically CELMs contained a low amount of the total protein content ( $1.5 \pm 0.6\%$ ,  $n=9$ ). Distribution of SM and Cer in the sucrose density fractions was also examined:  $64 \pm 8\%$  ( $n=4$ ) and  $49.7 \pm 5\%$  ( $n=4$ ) of total labeled SM and Cer, respectively, were detected in CELMs (Fig. 1C). According to the high SM content, CELMs were characterized also by a significant relative amount of cholesterol ( $43.7 \pm 6\%$  ( $n=3$ ) of the total labeled cholesterol) (Fig. 1D).

On the basis of the data obtained by the analysis of cav and lipid level distribution, in the subsequent experiments fractions 2–5, 7–9 and 10–13, corresponding to CELMs, intermediate (IM) and high density (HM) membranes, respectively, were pooled. Distribution of neutral CDase as well as acid and neutral SMase activities in membrane fractions was then examined. As it can be observed in Fig. 2A, neutral CDase was detectable in CELMs; the specific activity of the enzyme in these microdomains was substantially comparable to that measured in HMs. In agreement, in CELMs, as well as in the other fractions, Western analysis of neutral CDase revealed a band of approximately 94 kDa. In addition, neutral CDase seems to associate with cav in CELMs since CDase activity was detectable in anti-cav immunoprecipitates (data not shown).

Notably, acid (Fig. 2B) and neutral (Fig. 2C) SMase activities were present in CELMs, both at a higher specific activity

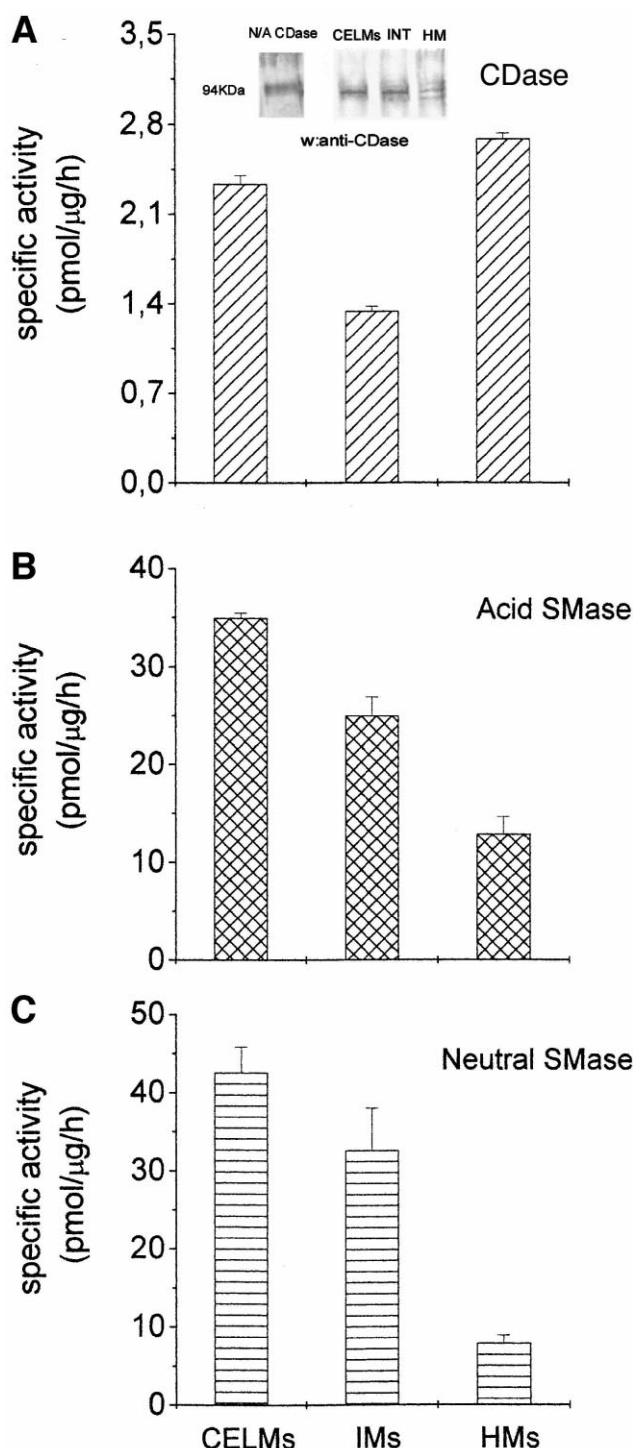


Fig. 2. CDase and SMase activities in sucrose density gradient fractions. Sucrose density gradient fractions corresponding to 2–5 (CELMs), 6–9 (IMs) and 10–13 (HMs) were pooled. Samples were assayed for neutral CDase activity (A) and subjected to Western analysis using specific anti-neutral CDase antibodies (A, inset). Recombinant mouse liver CDase was used as control. Acid SMase (B) and neutral SMase (C) activities were also measured. Data are means  $\pm$  S.E.M. of a representative experiment of at least three performed in triplicate.

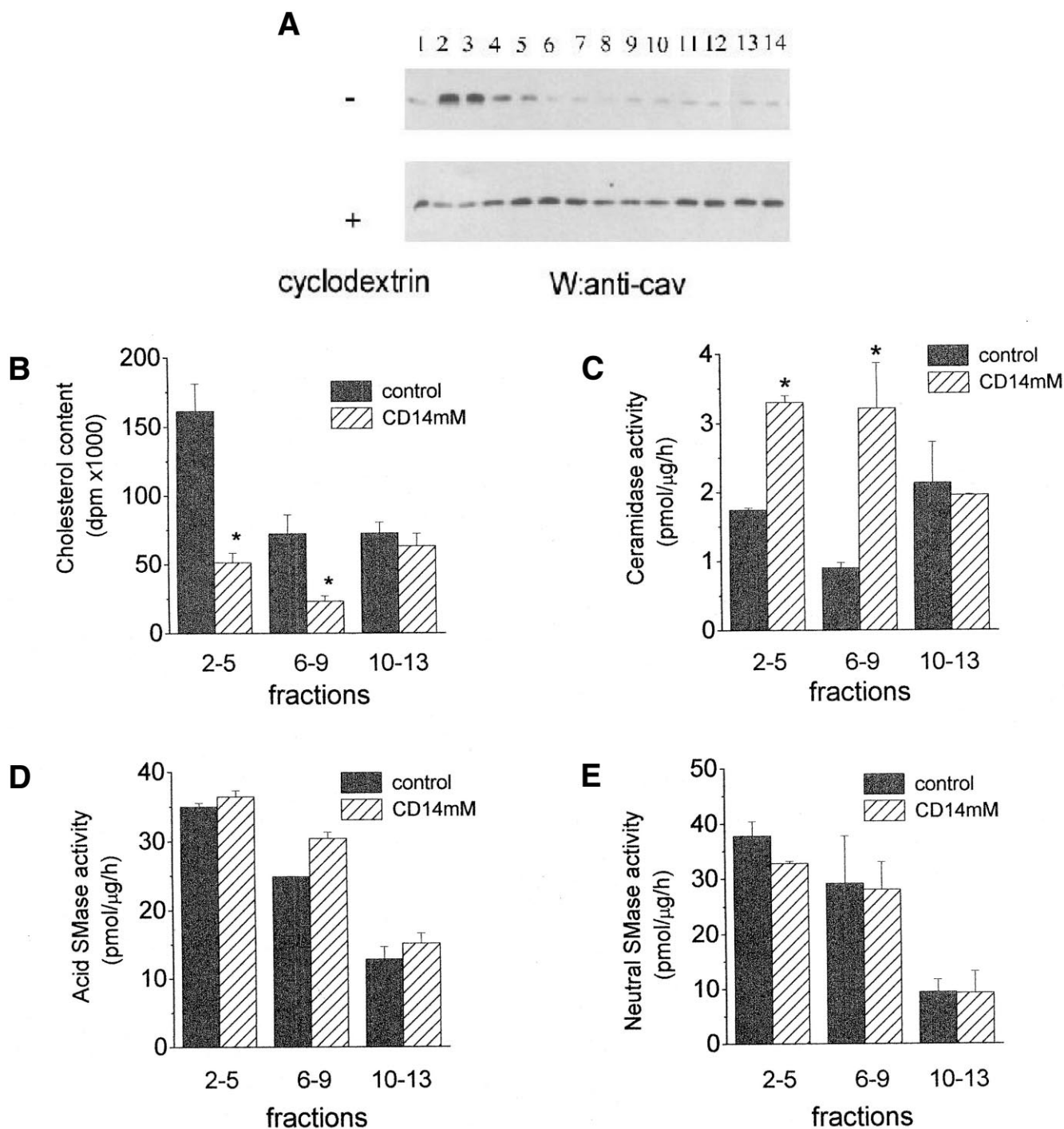


Fig. 3. Effect of cholesterol depletion on cav distribution, CDase and SMase activities. Confluent H.end cells were treated with 14 mM CD for 1 h and processed for sucrose density gradient fractionation. Samples of each fraction from control (–) and CD-treated (+) cells were analyzed for the presence of cav (A). Cholesterol content (B), neutral CDase (C), acid (D) and neutral (E) SMase activities were also measured in control and CD-treated cells. Data are means  $\pm$  S.E.M. of a representative experiment of three performed in triplicate. When not shown the error bar lies within the column.

than that measured in the other membrane fractions. Neutral SMase activity in CELMs was not activated by the addition of  $\text{MgCl}_2$  to the enzyme assay mixture ( $42.6 \pm 6.3$  and  $48.2 \pm 5.1$  pmol/μg/h in the absence or presence of 10 mM  $\text{MgCl}_2$ , respectively ( $n=3$ )), suggesting that a  $\text{Mg}^{2+}$ -independent isoform is located in these microdomains.

Cell treatment with CD has been successfully utilized in various instances to deplete cholesterol. As reported in Fig.

3, incubation of H.end cells for 1 h with 14 mM CD efficiently removed cholesterol from membranes. Cholesterol depletion was responsible also for a profound redistribution of the caveolae marker cav, which was detectable in all gradient fractions (Fig. 3A). Remarkably, a significant rise in neutral CDase activity in light membranes and IMs was observed in CD-treated cells (Fig. 3C), suggesting a negative role of cholesterol in CDase regulation. Consistently, in HMs, where

cholesterol levels were not affected by CD treatment, no significant variation of CDase activity was detected. On the contrary, neither neutral nor acid SMase activity was affected by cholesterol depletion (Fig. 3D,E), indicating a differential sensitivity of murine endothelial cell CDase and SMase activities to cholesterol.

#### 4. Discussion

In this report we have shown that CELMs from murine endothelial cells, enriched in SM, Cer and cholesterol, bear neutral and acid SMase activities as well as neutral CDase, suggesting that in these microdomains active SM metabolism can take place. Acid SMase was already described in light detergent-insoluble membranes [14] and more recently neutral and acid SMase activities were found associated to these microdomains [18].

In this study evidence is presented that also CELMs obtained from H.end endothelial cells contain acid and neutral SMase activities. However, differently from what is observed in sphingolipid-enriched microdomains of human fibroblasts [18], neutral SMase localized in CELMs of endothelial cells resulted to be  $Mg^{2+}$ -independent, suggesting that a distinct SMase isoform is present. This hypothesis is further supported by the different sensitivity to cholesterol depletion of neutral SMase activity in the two cell systems, given that endothelial neutral SMase localized in membrane fractions, in disagreement with fibroblast SMase [18], was not affected by treatment with CD.

Here is reported for the first time that neutral CDase is localized in CELMs of endothelial cells. Of note, CDase was found distributed among sucrose density fractions differently from acid and neutral SMase activities. Indeed, specific activity of CDase in CELMs was comparable to that detected in HMs, whereas specific activities of neutral and acid SMase were by far higher than those of the other fractions.

The identification of CDase in CELMs adds a new piece of information to the comprehension of subcellular distribution of CDases. Indeed, overexpressed human neutral CDase was detected in mitochondria of MCF7 and HEK293 cells [8], more recently immunofluorescence studies identified mouse liver neutral CDase mainly in late endosomes/lysosomes while rat kidney neutral CDase resulted to be almost exclusively in lipid rafts of apical membranes [9]. Endothelial cell neutral CDase was almost completely solubilized by freeze-thawing (Romiti et al., unpublished results), similarly to what was observed for mouse liver enzyme [31], thus suggesting that the bulk of the enzyme is located in intracellular vesicles. In agreement with this hypothesis, H.end cells actively release neutral CDase in the medium [27]. The identification of neutral CDase in CELMs is not in disagreement with a mainly vesicular membrane localization of the enzyme in endothelial cells, given that proteins located in CELMs represented less than 2% of total cell protein. Another interesting finding of this study is represented by the large stimulatory effect exerted by cholesterol depletion on CDase activity. Cholesterol depletion is known to alter caveolar structure [12] and this experimental approach has been utilized in several instances to ascribe eventual observed phenomena to destruction of membrane microdomains and/or to prove specific interaction with the caveolae scaffolding protein cav. Here we provided experimental evidence that cholesterol depletion of H.end cells was indeed responsible for a redistribution of cav throughout the

sucrose gradient fractions, as also observed in other experimental settings [32]. However, the finding that CDase activity was significantly increased by cholesterol depletion also in IMs, where CD treatment increased cav content, is consistent with a specific regulatory role of CDase exerted by cholesterol. The importance of this newly discovered CDase regulatory mechanism for the maintaining and fate of caveolae, known to be involved in cell cholesterol efflux in this cell type [33], remains to be established, although it is suggestive to speculate that CDase may have a role in lipid remodeling subsequently to cholesterol delivery by caveolae.

In addition to a possible role of caveolar neutral CDase in the dynamics of these microdomains, participation of the enzyme in signaling events compartmentalized in caveolae can also be postulated. Since neutral CDase is known to be up-regulated by extracellular ligands, such as platelet growth factor [34], it will be of interest to investigate whether caveolar CDase activity is agonist-regulated given that an important role of caveolar Cer has already been proved in the control of the phosphatidylinositol 3-kinase survival pathway [35].

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