BENE, a Novel Raft-associated Protein of the MAL Proteolipid Family, Interacts with Caveolin-1 in Human Endothelial-like ECV304 Cells*

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The MAL proteolipid, an integral protein present in glycolipid- and cholesterol-enriched membrane (GEM) rafts, is an element of the machinery necessary for apical sorting in polarized epithelial Madin-Darby canine kidney cells. MAL was the first member identified of an extended family of proteins that have significant overall sequence identity. In this study we have used a newly generated monoclonal antibody to investigate an unedified member of this family, named BENE, which was found to be expressed in endothelial-like ECV304 cells and normal human endothelium. Human BENE was characterized as a proteolipid protein predominantly present in GEM rafts in ECV304 cells. Coimmunoprecipitation experiments revealed that BENE interacted with caveolin-1. Confocal immunofluorescence and electron microscopic analyses indicated that BENE mainly accumulated into intracellular vesicular/tubular structures that partially colocalize with internal caveolin-1. In response to cell surface cholesterol oxidation, BENE redistributed to the dilated vesicular structures that concentrate most of the caveolin-1 originally on the cell surface. After cessation of cholesterol oxidation, a detectable fraction of the BENE molecules migrated to the plasmalemma accompanying caveolin-1 and then returned progressively to its steady state distribution. Together, these features highlight the BENE proteolipid as being an element of the machinery for raft-mediated trafficking in endothelial cells.

The compartmentation of cellular membranes in microdomains or rafts is an emerging concept in cell biology (1). Unlike the bulk of membranes, which are enriched in phospholipids and packed in a disordered state, rafts have a high glycolipid-glycolipid and cholesterol content and appear to be packed in a liquid-ordered structure (2). This difference makes glycolipid- and cholesterol-enriched membrane (GEM) rafts resistant to solubilization by nonionic detergents at low temperature (2). Recruitment of specific proteins into rafts was initially proposed to explain the segregation and transport of apical proteins during biosynthetic transport in polarized epithelial cells (3). More recently, this model has been extended as a general mechanism for protein recruitment in a variety of cellular processes including membrane trafficking and signaling (1). Although their characteristic lipid composition provides the biophysical basis for the specificity of protein recruitment by compatibility with the raft structure, it is believed that rafts require protein machinery to be operative in signaling or transport (1, 3).

Caveolae are raft-containing vesicular invaginations of the plasma membrane involved in a variety of cellular processes including signaling and clathrin-independent endocytosis (4). Caveolin-1 is a multifunctional raft-associated protein (5) primarily identified as a component of the caveolar architecture (6). Caveolin-1 is believed to be an element of the protein machinery operating in rafts, because: 1) it is able to direct the organization of rafts in caveolae-like vesicles (7, 8), and 2) it forms a scaffold onto which many classes of signaling molecules can assemble to generate preassembled signaling complexes within caveolae (5). The existence of a family of proteins similar to caveolin-1 with at least two other proteins, termed caveolin-2 and caveolin-3, which are resident in GEMs, suggests that members of the caveolin family are elements of the machinery involved in raft organization (5). The flotillin/cavatellin family, which so far groups the raft-associated flotillin-1 and flotillin-2/ESA proteins (9), whose function is still unknown, might constitute a second family of elements of the raft machinery (5).

Proteolipids are operationally defined as proteins with unusually high solubility in organic solvents commonly used to extract cell lipids (10). MAL is an integral membrane proteolipid protein of 17 kDa expressed in a restricted range of cell types including polarized epithelial cells (11, 12), oligodendro-

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1 The abbreviations used are: GEM, glycolipid- and cholesterol-enriched membrane; CO, cholesterol oxidase; MDCK, Madin-Darby canine kidney; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; GPI, glycosylphosphatidylinositol.
cytes (13), and T lymphocytes (14, 15). MAL selectively resides in lipid rafts in all the cell types in which it is expressed (11–14). An essential role for MAL in apical sorting has recently been demonstrated by the observation that depletion of endogenous MAL severely reduces the overall transport of membrane proteins to the apical surface in polarized epithelial Madin-Darby canine kidney (MDCK) and Fischer rat thyroid cells (16–18). This highlights MAL as a component of the machinery acting in the organization of rafts for apical transport. The presence in the GenBank™ of cDNAs encoding for proteins with significant overall sequence identity with MAL was indicative of the existence of a family of proteins related to MAL, henceforth referred to as the “MAL family” of proteins (19, 20). The demonstrated role of MAL as an element of the raft machinery in epithelial cells is consistent with the early proposal that the MAL family of proteolipid proteins might be involved in raft organization (19).

The observation that GEMs are resistant to solubilization in nonionic detergents at low temperatures has been widely exploited for the biochemical isolation of a membrane fraction that appears to be derived from cellular rafts (21). So far, no member of the MAL family of proteolipid proteins has been identified in the GEM fraction of endothelial cells (22). The MAL gene, a member of the MAL family gene, was originally cloned during a search for genes present in the vicinity of the human immunoglobulin κ chain locus (23). BENE mRNA is expressed in the prostate, small intestine, colon, heart, and lung and is undetectable in brain, thymus, liver, and spleen (20). In this study, using a newly developed anti-BENE monoclonal antibody (mAb) we have identified endogenous BENE in the GEM fraction of ECV304 cells, a human cell line displaying endothelial-like features (24). We have detected a physical interaction between BENE and caveolin-1 and observed a partial colocalization between these two proteins in vesicular/tubular structures in ECV304 cells. Oxidation of surface cholesterol by cholesterol oxidase (CO) and cessation of that process by treatment with 0.5 mg/ml G418 sulfate (Life Technologies, Inc.) for at least 4 weeks following transfection. Drug-resistant cells were selected, screened by immunofluorescence analysis with 9E10 mAb, and the clones that proved to be positive for tagged BENE expression were maintained in drug-free medium. After several passages in this medium >90% of cells within the selected positive clones retained expression of tagged BENE. The MDCK cell stable transfectants expressing tagged BENE used for the hybridoma screening were generated following an identical procedure.

Preparation of Monoclonal Antibodies to Human BENE—The peptide EKLLDPRPIYY1, corresponding to amino acids 118–128 of the human BENE molecule, was synthesized in an automated multiple peptide synthesizer (AMS 422, Abimed, Langerfeld, Germany) using the solid phase procedure and FMOC-N(N-9-fluorenylmethoxycarbonyl) chemistry (26). After coupling to keyhole limpet hemocyanin, the peptide was used to immunize Wistar rats. Spleen cells from immunized rats were fused to myeloma cells following standard protocols (27) and plated onto microtiter plates. The culture supernatants were screened by immunoblot analysis using BENE-enriched membrane fractions prepared from epithelial MDCK cells that stably expressed the BENE protein tagged with the 9E10 c-Myc epitope. The hybridoma clone 5B1, which secretes antibodies to human BENE, was isolated after several rounds of screening and used to produce culture supernatants containing 5B1 mAb.

Northern Blot Analysis—Total RNA from different cell lines was extracted using the Ultraspec RNA isolation system (Biotex Laboratories, Houston, TX). For Northern blot analysis, 10–20 μg of RNA were denatured in 50% formamide and 2.2 M formaldehyde gel, and transferred to Nylon membranes. RNA samples were hybridized under standard conditions to cDNA fragments labeled by the random-primer method (28) corresponding to human BENE (23). As a control of the amounts of RNA present in each lane, blots were finally hybridized with a 0.6-kilobase pair HindIII/BamHI DNA fragment from the 3′-untranslated region of human β-actin mRNA (29). Final blot washing conditions were 0.5 × SSC/0.1% SDS (1 × SSC = 0.15 mM NaCl, 5 mM EDTA, 1% Triton X-100) at 4 °C. The lystate was scraped from the dishes with a cell lifter, the dishes were rinsed with 1 ml of the same buffer at 4 °C, and the lystate was homogenized by passing the sample through a 22-gauge needle. The lystate was finally brought to 40% sucrose (w/w) in a final volume of 4 ml and placed at the bottom of an 8-ml 5–30% linear sucrose gradient. Gradients were centrifuged for 18 h at 39,000 rpm at 4 °C in a Beckman SW41 rotor. Fractions of 1 ml were harvested from the bottom of the tube, and aliquots were subjected to immunoblot analysis. Density was determined by measuring the refractive index of the fractions. In some experiments, centrifugation to equilibrium was carried out using discontinuous sucrose density gradients consisting of a 4-ml bottom layer containing the cell lystate at 40% sucrose, overlaid with 6 ml of 30% sucrose and a 2-ml layer of 5% sucrose at the top. After centrifugation, the opalescent band containing GEMs, which migrates in the 5–30% sucrose interphase, was harvested from the top (fraction I). The 40% sucrose layer containing the cytosolic proteins and the solubilized proteins was also harvested (fraction S).

Immunoblot and Immunoprecipitation Analyses—For immunoblot analysis, samples were subjected to SDS-PAGE in 10% acrylamide gels and transferred to nitrocellulose membranes (Millipore, Bedford, MA). After blocking with 5% nonfat dry milk, 0.05% Tween 20 in phosphate-buffered saline, blots were incubated with the indicated primary antibody. After several washings, blots were incubated for 1 h with secondary goat anti-IgG antibodies coupled to horse-radish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham...
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Pharmacia Biotech. For immunoprecipitation studies, cells were incubated for 4 h at 4 °C with a control antibody bound to protein G-Sepharose, and the supernatant immunoprecipitated by incubation for 4 h at 4 °C with the immunoaffinity-bound to protein G-Sepharose. Immunoprecipitates were washed six times with 1 ml of 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100 and analyzed by SDS-PAGE under reducing conditions. To detect 

RESULTS

Expression of the BENE Gene in Different Cell Lines—A partial BENE cDNA was identified during a search for genes in the proximity of the human immunoglobulin κ chain locus (23). This cDNA clone (EBI/GenBank™ data library accession number U17077) has an open reading frame of 148 amino acids showing ~39% identity with the MAL protein sequence but lacks an in-frame ATG triplet that could be used as a translational initiation codon. During a search of the TIGR Human Gene Index we identified a partial cDNA clone that both matched the BENE sequence and contained an additional 5'-upstream sequence (EBI/GenBank™ data library accession number D83824). This additional sequence displays a unique ATG codon. During a search of the TIGR Human Gene Index we identified a partial cDNA clone that both matched the BENE sequence and contained an additional 5'-upstream sequence (EBI/GenBank™ data library accession number D83824). This additional sequence displays a unique ATG codon.

Immunohistochemical Analysis—Human tonsils were received as routine specimens obtained from surgery. Samples were fixed for several hours in 10% neutral buffered formalin and subjected to routine tissue processing and paraffin embedding. Sections of 5-μm thickness were prepared from paraffin-embedded tissues and were mounted on polyl-lysine-coated glass microslides. Antigen retrieval was accomplished by subjecting deparaffinized sections to microwave unmasking for 60 s in 200 mM citrate buffer, pH 6.0. The tissue was then blocked with a 1:20 dilution of normal rabbit serum in 10 mM Tris-HCl saline buffer, pH 7.6, as described previously (34). The sections were sequentially incubated with a 1:100 dilution of an ascites stock of anti-BENE 5B1 mAb and peroxidase-conjugated rabbit anti-rat IgG (Bio-Rad). Each incubation was followed by washes with Tris-buffered saline. Then, sections were incubated with Graham-Karnovsky medium containing 0.5 mg/ml of 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and hydrogen peroxide. Sections were counterstained with Carazzi's hematoxylin, dehydrated, and mounted by routine methods.

Fig. 1. Alignment of the human BENE and MAL protein sequences. The sequence of BENE was reconstituted by adding the NH2-terminal sequences encoded by a partial expressed sequence tag cDNA clone (GenBank™ accession number D83824) (underlined with a dashed line) to the predicted open reading frame of the previously reported incomplete BENE cDNA (GenBank™ accession number U17077). The position of three different sequences in the BENE protein that fit with consensus sequences (φ-X-φ-X-X-X-φ; φ-X-X-φ-X-φ-φ-φ, where φ stands for an aromatic amino acid and X for any amino acid) of interaction with the scaffolding domain of caveolin-1 are indicated. The sequence of the peptide used to produce antibodies to BENE is underlined with a continuous line. The amino acids in identical positions in the BENE and MAL sequences are boxed.
and the product was cloned and sequenced. The amino acid sequence predicted from this analysis was identical to that shown in Fig. 1.

**Generation and Characterization of a Monoclonal Antibody to the BENE Protein**—The peptide EKLLDPRIYYI, comprising amino acids 118–128 of human BENE, was synthesized (sequence underlined in Fig. 1), coupled to keyhole limpet hemocyanin, and used to immunize Wistar rats. The selected peptide is located in the BENE molecule in a position equivalent to that of the MAL peptide previously used to generate anti-MAL antibodies (12, 16), in which MAL corresponds to an extracellular (luminal) loop (36). A hybridoma clone (named 5B1) producing antibodies to BENE was identified by immunoblot analysis of membrane fractions enriched in tagged BENE obtained from transfected MDCK cells. The 5B1 mAb specifically identified a protein band of the predicted size in COS-7 cells transiently expressing tagged BENE but not in untransfected cells (Fig. 3A). The BENE peptide used for the immunizations, at concentrations of 500 ng/ml, was able to totally neutralize the recognition of BENE by mAb 5B1, whereas other control peptides did not present any effect (not shown). The observed effect was specific for the 5B1 mAb, since the same peptide did not influence the recognition of tagged BENE by anti-c-Myc 9E10 mAb (Fig. 3B). As a further test of the specificity of the 5B1 mAb, Fig. 3C shows that the antibody recognized endogenous BENE in ECV304 and A498 cells, which were positive for BENE mRNA expression, but not in the Jurkat T cell line (Fig. 3C) or HepG-2 cells (not shown), which lack BENE mRNA (Fig. 2). The endogenous BENE protein migrated with the same electrophoretic mobility as the endogenous MAL protein from Jurkat cells, as would be expected given the absence of MAL transcripts in the Jurkat cells, as would have been expected given that the length of the two proteins was calculated to be equal. In addition, Fig. 3C shows that ECV304 cells lack detectable expression of MAL, as would be expected given the absence of MAL transcripts in this cell line. Although ECV304 cells display some endothelial features (24), the endothelial nature of this cell line has been questioned recently (37). To examine whether BENE is expressed in normal endothelia, human tonsil sections were subjected to immunohistochemical analysis with anti-BENE mAb 5B1. As shown in Fig. 3D, in agreement with the presence of BENE in the endothelial-like ECV304 cell line, BENE staining was detected in the endothelial layer lining the blood vessels.

**Endogenous BENE Is a Proteolipid Protein Present in GEM Microdomains in Endothelial ECV304 Cells**—The GEM fraction, which is resistant to solubilization by nonionic detergent at low temperatures, was separated from the bulk of cellular membranes, which are solubilized by the detergent, and from cytosolic proteins by using an established protocol involving centrifugation to equilibrium on sucrose density gradients (21). After fractionation from the top of the gradient, aliquots from each fraction were subsequently separated by SDS-PAGE and immunoblotted with mAb 5B1. Fig. 4A shows that endogenous BENE was found selectively in the GEM fraction of ECV304 cells. As controls we observed that the same fraction contained caveolin-1 and caveolin-2, two proteins already described in GEMs, but did not contain calnexin, a transmembrane protein present in the endoplasmic reticulum. To investigate whether BENE displays lipid-like properties, as is the case with MAL, the GEM fraction from ECV304 cells was extracted with n-butyl alcohol, and after phase separation, the resulting aqueous and organic phases were analyzed by immunoblot with anti-BENE mAb 5B1 and counterstained with hematoxylin to visualize nuclei. Reactivity was found in endothelial cells lining the blood vessels (arrows).

**Fig. 2.** Expression of the BENE gene in different cell lines. Total RNA (~20 μg) from the indicated cell lines was hybridized to DNA probes specific to BENE or β-actin.

**Fig. 3.** Characterization of a novel monoclonal antibody to human BENE. A, immunoblot analysis of the anti-BENE 5B1 mAb. The hybridoma clone 5B1 producing mAb to the human BENE protein was isolated after screening of the hybridoma culture supernatants. To assay the specificity of mAb 5B1, protein extracts from untransfected (—) or from transfected COS-7 cells transiently expressing BENE tagged with the c-Myc 9E10 epitope (BENE) were subjected to immunoblot analysis with either mAb 5B1 mAb or with the anti-tag mAb 9E10. As COS-7 cells are negative for BENE gene expression (not shown), no reaction was observed with endogenous proteins of COS-7 cells. B, to further study the specificity of the 5B1 mAb, aliquots of 5B1 culture supernatant were preincubated for 1 h at 4 °C with the indicated amounts of the BENE peptide used for the immunizations and used to probe blots of extracts from COS-7 cells transiently expressing the human BENE protein tagged with the c-Myc 9E10 epitope. Other unrelated peptides used did not show any effect on the recognition of BENE by the 5B1 mAb (not shown). The same blots were then reprobed with anti-c-Myc 9E10 mAb preincubated with the BENE peptide to show that the competition observed with the 5B1 mAb was specific. Note that similar amounts of tagged BENE were present in each lane. C, mAb 5B1 detects endogenous BENE in endothelial ECV304 cells. Extracts from ECV304 cells and Jurkat T cells were subjected to immunoblot analysis with anti-BENE 5B1 mAb and anti-MAL 6D9 mAb as indicated. D, tonsil sections were subjected to immunohistochemical analysis with anti-BENE mAb 5B1 and counterstained with hematoxylin to visualize nuclei. Reactivity was found in endothelial cells lining the blood vessels (arrows).

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BENE mAb 5B1 of a pool of the insoluble membrane fractions from ECV304/BENE cells (Fig. 4D).

**BENE Is Associated with Caveolin-1 and -2 in ECV304 Cells**—Lipid rafts, such as those containing caveolae or GPI-anchored proteins, coalesce after detergent extraction, making impossible the distinction between different types of rafts (2). The presence of BENE in GEM raft fractions led us to carry out a comparative immunofluorescence analysis of the distribution of caveolin-1, the GPI-anchored CD59 molecule, and BENE in ECV304 cells. As the anti-BENE 5B1 mAb is not of use for immunoprecipitation studies, to gate the relationship between BENE and caveolin-1, we carried out an association of caveolin-1 with exogenous BENE using ECV304/BENE cells. The 5B1 mAb is not of use for immunoprecipitation studies, to carry out the reciprocal experiment we used anti-tag antibodies to immunoprecipitate exogenous BENE from extracts obtained from transfected ECV304/BENE cells. Fig. 6B shows that, in addition to tagged BENE, the anti-tag antibodies immunoprecipitate two protein bands from metabolically labeled ECV304/BENE cells. These are -22–24 kDa, which corresponds to the size of caveolins. The presence of caveolin-1 in the BENE immunoprecipitate was confirmed by immunoblotting with anti-caveolin-1 antibodies (Fig. 6C). Caveolin-1 is known to interact with caveolin-2 to form heterooligomers (39), raising the possibility that caveolin-2 was in the same complex. The presence of caveolin-2 in the BENE immunoprecipitates was demonstrated by immunoblotting with anti-caveolin-2 antibodies (Fig. 6C).

**Distribution of BENE in ECV304 Cells**—To investigate the distribution of BENE we carried out immunoelectron microscopy on ultrathin cryosections with anti-tag antibodies using ECV304 cells stably expressing tagged BENE. BENE was localized in small tubular-vesicular elements scattered throughout the cell (Fig. 7). These immunoreactive profiles were also observed in the Golgi region (Fig. 8a and b). Occasionally, Golgi cisterna and buds were also labeled (Fig. 8b). No labeling was detected in other cytoplasmic organelles or the plasma membrane (Fig. 8c). The membranes containing BENE appeared on an ultrathin section as 50–70 nm vesicular profiles or as short nonbranching tubules (Fig. 8, c and d, inset). These membranes were occasionally covered by a characteristic 18-nm-thick coat (Fig. 7, inset), which has been unambiguously identified in previous studies as being made of clathrin (30, 33). To investigate the relationship between BENE and caveolin-1, we carried out a comparative analysis of the distribution of these proteins.
Membranes immunolabeled for BENE were screened for the presence of caveolin-1 or a clathrin coat using the procedure described under "Experimental Procedures." The quantitative analysis showed that 86.2% of BENE colocalized with caveolin-1 in the same uncoated membranes (Fig. 8, c and d), in agreement with our results showing a physical interaction between these proteins. Quantitative analysis indicated that most of the remaining BENE molecules are associated with cytoplasmic tubular/vesicular structures that lack a discernible coat (88.6% of the total reactive vesicles) and a small fraction is associated with similar structures displaying a typical clathrin coat (4.6% of the total labeling). The caveolin-1 associated with the structures containing BENE represented 10% of the total caveolin-1.

Effect of Cholesterol Oxidation on the Distribution of BENE—There is a rapid redistribution of surface caveolin-1 to the Golgi region in response to surface cholesterol oxidation by extracellular CO (25). As both BENE and caveolin-1 reside in insoluble lipid rafts, we employed confocal immunofluorescence to establish whether BENE distribution is also sensitive to cholesterol oxidation using ECV304/BENE cells and anti-tag antibodies. Optical sections were taken at 0.4-μm intervals along the z axis of the cells at different times of CO treatment using an optimum pinhole. For simplicity only two sections are shown illustrating either the perinuclear region and the plane of the plasma membrane just underneath the nucleus (a) or to the periphery of the cell, also including part of the perinuclear region (b). Fig. 9 shows that at steady-state (untreated cells) BENE was mostly found in small discrete structures in the perinuclear region (a) with little labeling at the cell periphery (b). Although most of the caveolin-1 labeling was on the cell surface, caveolin-1 was also found in the perinuclear region as described previously (40). A significant fraction of the internal caveolin-1 colocalized with BENE, consistent with our electron microscopic results. In response to cholesterol oxidation, the distribution of BENE progressively switches from its steady-state distribution in a large number of small vesicular profiles to become concentrated into a reduced number of structures with a dilated appearance. After 30 min of CO treatment, this
Redistribution of BENE was already detectable (a), the typical accumulation of surface caveolin-1 at the leading edge had been lost (a), and caveolin-1 had begun to be internalized (a and b). After 60 min of CO treatment, most of the BENE accumulated into dilated structures, and caveolin-1 had been fully internalized. It is of particular note that, under these conditions, internalized caveolin-1 was totally concentrated in the dilated structures stained for BENE.

It has been established that after cessation of cholesterol oxidation, caveolin-1 returns to the cell surface, probably to replenish the plasma membrane with fresh cholesterol (25). Fig. 10 shows that 10 min after CO withdrawal BENE already no longer appeared in the dilated structures observed in CO-treated cells but rather reacquired a discrete vesicular pattern. Simultaneously, most of the caveolin-1 originally in the Golgi complex (G), lateral rims of Golgi cisterna were occasionally immunoreactive (arrows), note the high labeling for caveolin-1 in a cluster of intracellular caveola-like vesicles. The arrowhead points to a vesicle immunoreactive for BENE and caveolin-1. d, note the high labeling for caveolin-1 in a cluster of intracellular caveola-like vesicles. The arrowhead points to a vesicle immunoreactive also for BENE. The inset in d shows a vesicle containing BENE and caveolin-1. n = nucleus; pm = plasma membrane; G = Golgi stack. Bars, 200 nm.

**Fig. 9.** Redistribution of BENE and caveolin-1 in response to cholesterol oxidase treatment in endothelial-like ECV304 cells. ECV304/BENE cells grown on coverslips were left untreated or treated with 1 unit/ml CO for the indicated times. Cells were fixed and processed for confocal immunofluorescence analysis with anti-tag mAb 9E10 and rabbit polyclonal antibodies to caveolin-1, followed by appropriate Texas Red-labeled and fluorescein-labeled secondary antibodies to visualize BENE and caveolin-1, respectively. Two different optical sections of the same cells, at 1.2 μm (a) and 2.0 μm (b) from the plane of the coverslips, are shown. NT, nontreated. Bar, 8 μm.

This movement of caveolin-1 to the plasmalemma was accompanied by the translocation of a fraction of the BENE to the cell periphery (see b panels). The translocated BENE molecules were mostly located underneath surface caveolin-1 (see inset in the merge profile), although a low level of colocalization between both proteins was also detected. After 20 min of CO removal the translocation of BENE was much more pronounced, and BENE was clearly detected in close proximity to surface caveolin-1. Finally, 1 h after CO removal BENE and caveolin-1 distributions were both as at steady state.

**DISCUSSION**

**BENE Is a Proteolipid Protein with Selective Residence in Rafts in Endothelial-like ECV304 Cells**—Previous work aimed at the systematic identification of protein components of GEMs from endothelial cells found this membrane fraction to be highly enriched in caveolin-1 and to contain GPI-anchored proteins, scavenger receptors for modified forms of low density lipoprotein (CD36 and RAGE), a large number of signaling molecules, and cytoskeletal elements (22). Although the presence of MAL and other proteolipid proteins with an apparent size in the range of 14–20 kDa has been described in GEMs from other cell types (11, 12), no proteolipid protein of the MAL family has so far been reported as being present in the GEM fraction of endothelial cells. The BENE protein was assigned to the MAL family on the basis of its significant amino acid sequence identity (39%) with MAL and the similar hydrophobicity profiles (19, 20, 23). The generation of a mAb specific to BENE has allowed the detection of endogenous BENE in normal human endothelial cells and its identification as a 17-kDa proteolipid protein with selective residence in the GEM fraction of the endothelial-like ECV304 cell line. Thus, endogenous BENE is the second member of the MAL family of proteolipid proteins to be identified in GEMs so far.

**BENE Associates with Caveolin-1 and -2**—The distribution of the rafts containing BENE was clearly different from the surface rafts containing caveolin-1 or GPI-anchored proteins. Caveolin-1 is a multifunctional protein that interacts with a wide variety of proteins through the so-called “scaffolding domain,” a 20-amino acid sequence proximal to the putative membrane insertion sequence (5). In MDCK cells, caveolin-1 is present as homooligomers and as heterooligomer complexes with caveolin-2 (39). The fact that BENE contains three different regions that fit consensus sequences known to interact with the scaffolding domain of caveolin-1, and the partial colocalization of BENE with caveolin-1 in internal rafts, led us to investigate the possible interaction between these proteins. Using fully solubilized extracts, we found that BENE associates with...
both caveolin-1 and -2 as demonstrated by coimmunoprecipitation experiments, whereas in agreement with our previous results (41), no association of MAL with caveolin-1 was found in parallel experiments in MDCK cells under the same stringent conditions of solubilization (results not shown). Thus, interaction with caveolins appears to be a specific feature of BENE not shared by all members of the MAL proteolipid family. At the electron microscopic level, BENE was localized in tubular/vesicular structures scattered throughout the cytoplasm and in the Golgi region. Approximately 8% of these structures were also positive for caveolin-1 as revealed by quantitative analysis. This indicates that BENE and caveolin-1 might cooperate in raft-mediated processes in endothelial cells. In addition to the structures containing caveolin-1, BENE was also identified in uncoated and clathrin-coated cytoplasmic tubular/vesicular elements lacking caveolin-1. This indicates that, in addition to cooperate with caveolin-1, BENE might also be involved in caveolin-1-independent functions mediated by lipid rafts. The distribution of BENE suggests that, similarly to MAL, which cycles between the cell surface, endosomes and the trans-Golgi network (36), BENE also moves between different intracellular compartments.

BENE, Caveolin-1, and Cholesterol Trafficking—Caveolin-1 moves from surface caveolae to large intracellular structures in response to cholesterol oxidation by CO (25). Those structures have been characterized previously by immunofluorescence and electron microscopic analyses as a distended Golgi apparatus (25). Upon CO removal, caveolin leaves the Golgi and returns to the cell surface. This caveolin-1 cycle appears to be similar to constitutive caveolin-1 cycling, which involves the sequential movement of caveolin-1 from surface caveolae to the endoplasmic reticulum, the endoplasmic reticulum-Golgi intermediate compartment, the Golgi, and surface caveolae (42). A role for caveolin-1 in transport of cholesterol from the endoplasmic reticulum to the cell surface has been proposed based on the knowledge: 1) that caveolin-1 binds cholesterol (43, 44), 2) that caveolin-1 expression in cells lacking endogenous caveolin-1 causes a 4-fold increase in the rate of delivery of newly synthesized cholesterol to the plasma membrane (45), and 3) of the response of caveolin-1 to cholesterol oxidation (25). The interaction between BENE and caveolin-1 in EC304 cells and their partial colocalization in the Golgi region led us to examine in parallel the effect of cholesterol oxidation on the distribution of BENE and caveolin-1. Our results showed that in response to cholesterol oxidation, BENE was redistributed from its steady-state distribution in a large number of small discrete vesicles and accumulated in a reduced number of structures with dilated morphology. The CO-triggered redistribution of caveolin-1 to the Golgi was accompanied by the conversion of caveolin-1 from a cytoplasmically oriented membrane protein at the cell surface to an intraluminal protein present in large perinuclear vesicles (25). This change in distribution is paralleled by the exit of caveolin-1 from lipid rafts, as evidenced by its loss of insolubility, and consistently with its luminal location (25). Interestingly, the insolubility of BENE did not alter after 1 h of CO treatment. This indicates that BENE was still embedded in membrane rafts in the dilated structures observed after CO treatment. Moreover, despite the extensive colocalization of caveolin-1 with BENE observed in CO-treated cells, the level of association of caveolin-1 with BENE relative to that of steady state, as assayed by coimmunoprecipitation experiments, did not increase. These results are consistent with the presence of BENE and caveolin-1 in the membrane and lumen, respectively, of the dilated structures observed in CO-treated cells. Finally, the migration of a detectable proportion of the BENE molecules to the plasmalemma accompanying the return of caveolin-1 to the plasma membrane, as is observed after cessation of cholesterol oxidation, suggests that BENE might play a role in cholesterol homeostasis and/or caveolin-1 transport to the cell surface. Thus, in agreement with our previous proposal (19) and the demonstrated role of MAL in raft-dependent apical transport (16–18), BENE and possibly the other members of the MAL family of proteolipids might constitute elements of the raft machinery for the specialized membrane trafficking pathways that exist in the different cell types.

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