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

Received for publication, October 25, 2000, and in revised form, February 28, 2001

Published, JBC Papers in Press, April 6, 2001, DOI 10.1074/jbc.M009739200

María del Carmen de Marco‡‡, Leonor Kremer‡, Juan P. Albar‡, José A. Martínez-Menárguez**, José Ballesta†, María Angeles García-López‡‡, Mónica Marazuela‡‡, Rosa Puertollano‡, and Miguel A. Alonso‡‡‡

From the ‡Centro de Biología Molecular “Severo Ochoa,” Universidad Autónoma de Madrid, ¶Department of Immunology and Oncology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Cantoblanco, 28049 Madrid, Spain, the †Departamento de Biología Celular, Facultad de Medicina, Universidad de Murcia, 30071 Murcia, Spain, and the ¶¶Departamento de Endocrinología, Hospital de la Princesa, 28006 Madrid, Spain

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-glycerolipid 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 caveole-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-
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 BENE 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 CO withdrawal indicate that BENE participates in cholesterol-regulated processes also involving caveolin-1 (25) in the endothelial-like ECV304 cell line.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse hybridoma producing mAb 9E10 (IgG1) to the human c-Myc epitope EQKLISEED was purchased from the American Type Culture Collection. Rabbit polyclonal antibodies to the c-Myc tag were from Santa Cruz Biotecnologies (Santa Cruz, CA). Mouse mAb MEM-43 (IgG2a) to CD59 was kindly provided by Dr. V. Horejsi (Institute of Molecular Genetics, Prague, Czech Republic). The rabbit polyclonal antibody to caveolin-1, and the mouse mAbs to caveolin-1, caveolin-2, and calnexin, were from Transduction Laboratories (Nottingham, United Kingdom). The anti-human MAL 6D9 mAb has been described previously (12). Peroxidase-conjugated secondary anti-IgG antibodies were supplied by Pierce. Fluorescein- and Texas Red-conjugated secondary antibodies were from Southern Biotech (Birmingham, AL). Protein A-gold conjugates were obtained from the Cell Biology of Utrecht University (Utrecht, The Netherlands). CO was purchased from Roche Diagnostics (Mannheim, Germany).

**Cell Culture Conditions, DNA Constructs, and Transfections**—Human ECV304 cells (kindly provided by Dr. J. Riese, Centro Nacional de Biotecnología, Madrid) and epithelial MDCK cells were grown on Petri dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.), penicillin (50 unit/ml), and streptomycin (50 μg/ml), at 37 °C in an atmosphere of 5% CO2/95% air.

An incomplete human BENE cDNA clone (a kind gift from Dr. H. G. Zachau, Institute of Physiological Chemistry, Munich, Germany) lacking the 5′ end coding region was subcloned into the polymerase chain reaction with specific oligonucleotide primers that anneal with the 5′ and 3′ ends of the BENE coding region in the template plasmid (23). In addition to the annealing sequence, the 5′ primer contained sequences required to reconstitute the entire coding region of the BENE cDNA in accordance with the additional ATG-containing 5′-upstream BENE sequence found in the EBI/GenBankTM data base (accession number D83824). To insert the 9E10 c-Myc epitope at the NH2 terminus of BENE, the reconstituted BENE cDNA was amplified with the same 3′ primer and a new 5′ end primer with sequences encoding the 9E10 c-Myc epitope placed between the first and second codons of the BENE cDNA coding region. After amplification under conditions that did not allow cloning into the pCR3.1 DNA eukaryotic expression vector (Invitrogen, Groningen, The Netherlands) to generate the pCR/BENE construct.

Transfection of ECV304 cells with pCR/BENE was carried out by electroporation using the Electro Cell Manipulator 600 equipment (BTX, San Diego, CA). Selection of stable transfectants was carried out by growth in 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 EKLLDPRIYIY, 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 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 (Biotecnologı ´a, Madrid) 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.

**Detergent Extraction Procedures**—GEMs were prepared essentially as described by Brown and Rose (21). ECV304 cells grown to confluence in 100-mm dishes were rinsed with phosphate-buffered saline and lysed for 20 min in 1 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 at 4 °C. The lysate 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 lysate was homogenized by passing the sample through a 22-gauge needle. The lysate was finally homogenized with 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 M NaCl, 0.015 M sodium citrate, pH 7.0) at 65 °C.

**Immunoblot and Immunoprecipitation Analyses**—For immunoblot analysis, samples were subjected to SDS-PAGE in 15% acrylamide gels and transferred to nitrocellulose filters. After blocking, the blots were incubated with goat anti-IgG antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blotting kit (ECL, Amersham...
Pharmacia Biotech. For immunoprecipitation studies, cells were incubated for 4 h at 4 °C with a control antibody bound to protein G-Sepharose, centrifuged and the supernatant immunoprecipitated by incubation for 4 h at 4 °C with the indicated specific antibodies 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 35S label-blocking 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 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 developed with Graham-Kid socynsky 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.

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™ database 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™ database accession number D83824). This additional sequence displays a unique ATG codon in-frame with 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-Φ; Φ-X-X-X-Φ-X-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.

Plasmids. For transfection of ECV304 cells with BENE cDNA, cells were plated for 4 h at 4 °C with a control antibody bound to protein G-Sepharose, centrifuged and the supernatant immunoprecipitated by incubation for 4 h at 4 °C with the indicated specific antibodies 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 35S label-blocking 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 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 developed with Graham-Kid socynsky 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.

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™ database 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™ database accession number D83824). This additional sequence displays a unique ATG codon in-frame with the BENE open reading frame mentioned above. The sequence surrounding this ATG (AGCAGTG) is consistent with the A/GXXATGG consensus sequence (where X stands for any nucleotide) for optimal ATG translational start sites in eukaryotic cells (35). The reconstituted open reading frame predicts a protein of 153 amino acids containing an NH2-terminal five-amino acid extension compared with the incomplete sequence deduced previously (23). The complete sequence of BENE, with the additional residues underlined, and its alignment with the MAL protein are shown in Fig. 1. To identify a suitable model cell system for studying BENE, we carried out Northern blot analysis using a wide range of human cell lines. Fig. 2 shows that, in addition to the prostate carcinoma PC3 cell line from which the BENE cDNA was originally cloned (23), a full-length BENE mRNA band was present in renal epithelial A498 cells, in cervix carcinoma HeLa cells and in the endothelial-like ECV304 cell line. BENE transcrits were undetectable in the rest of the cell lines examined including T cells (Jurkat and HPB-ALL cells), epithelial MDCK cells, and hepatic HepG-2 cells. To identify BENE gene expression in ECV304 cells unambiguously, the BENE cDNA coding sequence was amplified by reverse transcriptase-polymerase chain reaction using total RNA from ECV304 cells,
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), which in 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 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.
c-Myc mAb 9E10. Fractions of 1 ml were collected from the bottom of the tube. Aliquots subjected to centrifugation to equilibrium in sucrose density gradients. (ECV304/BENE

ECV304/BENE

umbilical cells (HUVEC
densitometer. 
tagged BENE

and endogenous BENE were quantified in a exogenous (blotting with anti-BENE mAb 5B1. The signal corresponding to the fraction obtained from ECV304/BENE cells were analyzed by immuno-

A

B

C

Bottom

Top

1 2 3 4 5 6 7 8 9 10 11 12

BENE

Cav-1

Cav-2

Cav-1

Caveolin

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 caveoleae 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 only of use for immunoblotting, we used anti-tag antibodies and ECV304/BENE cells for immunolocalization studies. Fig. 5 shows that the rafts containing BENE were mostly intracellular and clearly different from those of surface caveoleae and CD59. Intracellular caveolin-1 and CD59 colocalized with BENE in structures in the Golgi region, as revealed with antibodies to the Golgi mannosidase II marker. This result indicated a possible functional and/or biochemical interaction between caveolin-1 and BENE in internal structures. The amino acid sequence of BENE contains three motifs that fit with consensus sequences implicated in interaction with caveolin-1 (Fig. 1) (38). To address the possible interaction of caveolin-1 with BENE, ECV304 cells were extracted with 1% Triton X-100 and 60 mM octyl glucoside, a procedure used to solubilize GEMs (21). After centrifugation, the supernatant containing solubilized GEMs was immunoprecipitated with either control or anti-caveolin-1 antibodies, and the immunoprecipitates were subjected to immunoblot analysis with anti-BENE 5B1 mAb. The left panel in Fig. 6A shows that endogenous BENE was specifically detected in the caveolin-1 immunoprecipitate, indicating an interaction between the two proteins. This association was also observed in A498 cells, which express higher levels of caveolin-1 (Fig. 6A, left panel). The association of caveolin-1 with BENE was corroborated by analyzing the association of caveolin-1 with exogenous BENE using ECV304/ BENE cells and anti-tag antibodies (Fig. 6A, middle panel). As 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). Fig. 6D shows an example of the efficiency of the solubilization procedure used to prepare the extracts for the immunoprecipitation experiments.

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% 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 (86% of the total reactive vesicles) and a small fraction is associated with similar structures displaying a typical clathrin coat (4% 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 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 area in CO-treated cells returned to the cell surface (a and b).

**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 distented 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 ectopic 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 plasma membrane 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.

**Acknowledgments**—We thank Dr. Carlos Sánchez for his expertise with the use of the confocal microscope.

**REFERENCES**

1. Simons, K., and Ikonen, E. (1997) *Nature* 387, 569–572.
2. Brown, D. A., and London, E. (2000) *J. Biol. Chem.* 275, 17221–17224.
3. Simons, K., and Wandinger-Ness, A. (1990) *Cell* 62, 207–210.
4. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.* 67, 199–225.
5. Smart, E. J., Graf, G. A., McNiven, M. A., Sessa, W. C., Engelman, J. A., Scherer, P. E., Okamoto, T., and Lisanti, M. P. (1999) *Mol. Cell. Biol.* 19, 7289–7304.
6. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y., Glenney, J. R., and Anderson, R. G. W. (1992) *Cell* 68, 673–682.
7. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 8655–8659.
8. Li, S., Song, K. S., Koh, S. S., Kikuchi, A., and Lisanti, M. P. (1996) *J. Biol. Chem.* 271, 28647–28654.
9. Volonté, D., Galbiati, F., Li, S., Nishiyama, K., Okamoto, and Lisanti, M. P. (1998) *J. Biol. Chem.* 274, 12702–12709.
10. Schlesinger, M. J. (1981) *Annu. Rev. Biochem.* 50, 193–206.
11. Zacchetti, D., Fournier, P., Murata, M., Fiedler, K., and Simons, K. (1995) *FEBS Lett.* 377, 465–469.
12. Martin-Belmonte, F., Kremer, L., Albar, J. P., Maranuela, M., and Alonso, J. A. (1998) *Endocrinology* 139, 2077–2084.
13. Kim, T., Fiedler, K., Madison, D. L., Krueger, W. H., and Pfeiffer, S. E. (1995) *J. Neurosci. Res.* 42, 413–422.
BENE, a Proteolipid of the MAL Family in Endothelial Cells

14. Millán, J., and Alonso, M. A. (1998) Eur. J. Immunol. 28, 3675–3684
15. Alonso, M. A., and Weissman, S. M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1997–2001
16. Puertollano, R., Martín-Belmonte, F., Millán, J., de Marco, M. C., Albar, J. P., Kremer, L., and Alonso, M. A. (1999) J. Cell Biol. 145, 141–145
17. Cheong, K. H., Zacchetti, D., Schneeberger, E. E., and Simons, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6241–6248
18. Martín-Belmonte, F., Puertollano, R., Millán, J., and Alonso, M. A. (2000) Mol. Biol. Cell 11, 2033–2045
19. Pérez, P., Puertollano, R., and Alonso, M. A. (1997) Biochem. Biophys. Res. Commun. 232, 618–621
20. Magyar, J. P., Ebensperger, C., Schaeeren-Wiemers, N., and Suter, U. (1997) Gene (Amst.) 189, 269–275
21. Brown, D. A., and Rose, J. K. (1992) Cell 68, 533–544
22. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tank, Z., Hermanowski-Vosatka, A., Tu, Y-H., and Sargiacomo, M. (1994) J. Cell Biol. 126, 111–126
23. BENE, a Proteolipid of the MAL Family in Endothelial Cells

2017

28. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 8–13
29. Poule, P., Gunning, P., Blau, H., and Kedes, L. (1983) Mol. Cell. Biol. 3, 1783–1791
30. Martín-Menárquez, J. A., Geuze, H. J., Slot, J. W., and Klumperman, J. (1999) Cell 98, 81–90
31. Slot, J. W., Geuze, H. J., Gigengack, S., Lienhard, G. E., and James, J. E. (1991) J. Cell Biol. 113, 123–135
32. Griffiths, G. (1993) Fine Structure Immunocytochemistry, Springer-Verlag, Berlin
33. MartínMenárquez, J. A., Geuze, H. J., and Ballesta, J. (1996) Eur. J. Cell Biol. 71, 137–143
34. Marazuela, M., Sánchez-Madrid, F., Acevedo, A., Larrañaga, E., and Landázuri, M. O. (1995) Clin. Exp. Immunol. 102, 328–334
35. Konak, M. (1996) Mamm. Genome 7, 563–574
36. Puertollano, R., and Alonso, M. A. (1999) Mol. Biol. Cell 10, 3435–3447
37. Brown, J., Reading, S. J., Jones, S., Fitchett, C. J., Howl, J., Martin, A., Longland, C. L., Michelangeli, F., Dubrova, Y. E., and Brown, C. A. (2000) Lab. Invest. 80, 37–45
38. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) J. Biol. Chem. 272, 6525–6533
39. Scheiffele, P., Verkade, P., Fra, A. M., Simons, K., and Ikonen, E. (1998) J. Cell Biol. 140, 795–806
40. Dupree, P., Parton, R. G., Raposo, G., Kurzchalia, T. V., and Simons, K. (1995) EMBO J. 14, 1597–1605
41. Millán, J., Puertollano, R., Fan, L., and Alonso, M. A. (1997) Biochem. Biophys. Res. Commun. 233, 707–712
42. Conrad, P. A., Smart, E. J., Anderson, R. G. W., and Bloom, G. S. (1995) J. Cell Biol. 131, 1421–1433
43. Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., and Simons, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10339–10343
44. Li, S., Song, K. S., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 568–573
45. Smart, E. J., Ying, Y.-S., Donzell, W. C., and Anderson, R. G. W. (1996) J. Biol. Chem. 271, 28427–28435