Examination of transcellular membrane protein polarity of bovine aortic endothelial cells in vitro using the cationic colloidal silica microbead membrane-isolation procedure

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Summary
In this report we describe a rapid, high-yield protocol for the isolation of apical (AP) and basolateral (BL) plasma membrane domains from monolayers of bovine aortic endothelial cells (BAECs) grown on tissue culture dishes as well as microcarrier beads. Using a modified cationic colloidal silica microbead membrane-isolation procedure, which deposits a uniform silica-polyacrylate pellicle over the entire AP membrane surface, a 4- to 9.6-fold relative enrichment of AP membrane and a 3.55- to 3.67-fold relative enrichment of BL membrane was obtained when the isolated domains were examined for silica and Na⁺/K⁺-ATPase, respectively. Immunoblotting of the isolated membrane domains displayed the presence of angiotensin-converting enzyme (ACE) exclusively in the AP domain and collagen receptors (CRs) highly enriched in the BL membrane domain when monolayers were grown on a gelatin substratum.

Key words: endothelial cell polarity, angiotensin-converting enzyme, collagen receptors, microcarriers, apical and basolateral membrane domains.

Introduction
Occupying a unique barrier position within the cardiovascular system, the endothelium simultaneously mediates bidirectional as well as specific vectorial molecular transport between the blood and interstitial fluids. Its luminal front presents a protective, non-thrombogenic, but highly interactive surface to the blood, while its abluminal aspect firmly anchors the cells to a variety of extracellular matrix (ECM) components and mediates molecular traffic from the underlying tissues. Given the extreme environmental differences with which the two membrane surfaces interface, the protein composition of the endothelial luminal (apical: AP) and abluminal (basolateral: BL) membranes maintains a high degree of PM protein asymmetry reflecting their specialized functional roles.

Biochemical and immunological studies have demonstrated that various types of endothelial cells possess polarized distribution of surface proteins in vitro (Jaffe et al. 1987; Muller and Gimbrone, 1986; Nakache et al. 1986; Ryan et al. 1976a,b, 1982) as well as in vivo (Becker and Harpel, 1976; Betz et al. 1980; Horvot et al. 1986; Ryan et al. 1976b; Vorbrodt, 1988). However, much of the information regarding PM protein asymmetry in vitro arises from studies on a variety of epithelial cell lines, especially Madin-Darby Canine Kidney (MDCK) cells (reviewed by Caplin and Matlin, 1989; Hubbard et al. 1989; Rodriguez-Boulan and Nelson, 1989; Simons and Fuller, 1985). Despite the polarized nature of endothelial cell monolayers, comparatively little data have been published on these important, specialized epithelia.

Using a modification of the cationic colloidal silica technique (Mason and Jacobson, 1985), we can quickly isolate and examine large quantities of high-yield AP and BL membrane domains of 3- to 9-fold enrichment from BAEC monolayers grown on gelatin-coated microcarriers or tissue culture dishes. As a result, we are able to identify domain-specific proteins without domain-specific labeling procedures or cumbersome cell fractionation and density gradient techniques.

Materials and methods
All reagents were obtained from Sigma, St. Louis, MO., unless indicated otherwise. All solutions were made with NANOpure water (Barnstead/Thermolyne, Dubuque, IA). All tissue culture procedures, wherever possible, were carried
out in a sterile transfer hood and all tissue culture solutions were filter sterilized through 0.22 μm cellulose acetate filters (Millipore, Bedford, MA). Hanks’ balanced salt solution containing Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS, Gibco, Grand Island, NY), pH 7.2, supplemented with 20 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES, U.S. Biochemicals, Cleveland, OH) was used in all procedures.

**Cells and cell culture**

Bovine aortic endothelial cells (BAEC) were isolated and cultured to ensure a homogeneous monolayer. Isolates were cultured in a complex mitogenic medium as described previously (Carson and Haudenschild, 1986; Stolz and Jacobson, 1991) for at least 3 passages so as to discourage proliferation of smooth muscle cells. After this time, BAEC could be transferred to a “simpler” medium consisting of Dulbecco’s Modified Eagle’s Medium (DME) supplemented with 10% fetal bovine serum (FBS) and antibiotics: 200 IU/ml penicillin G, 200 μg/ml streptomycin and 5 μg/ml amphotericin B. Cells were maintained on a substratum of 1% (w/v) gelatin (type I from swine skin, tissue culture grade) in phosphate buffered saline (PBS), pH 7.0, adsorbed to tissue culture dishes (Nunc-Vangard or Corning). Gelatin-coated tissue culture dishes were prepared as described previously (Stolz and Jacobson, 1991). BAEC cultures were confirmed as homogeneous using morphology, monolayer growth pattern and selective uptake of acetylated low-density lipoprotein labeled with a fluorescent dye (Di-I-Ac-LDL: BTI, Stoughton, MA) as described by Voyta et al. (1984). Confluent monolayers were subcultured using trypsin-EDTA to release the cells from the dish, then plated at a ratio of 1:2 to 1:4. Subcultures were fed every 2-3 days.

Membrane domains could not be effectively isolated if smooth muscle cell contamination or aberrant BAEC “sprouters” morphology were evident. The “sprouters”, previously described by Cotta-Pereira et al. (1980), grow between the monolayer of cells and the substratum. Their position, therefore, compromises monolayer-substratum adhesion and the entire sheet of cells is removed from the dish during the domain isolation procedure.

**Metabolic radiolabeling of endothelial cell cultures**

Endothelial cell proteins were metabolically labeled with \(^{35}\)S-methionine using a modification of a technique developed previously (Bravo, 1984). Confluent monolayers of endothelial cells were cultured in methionine-free DME (Flow Laboratories, Rockville, MD or Gibco, Grand Island, NY) supplemented with 20 μCi/ml \(^{35}\)S-methionine (Amersham, Arlington Heights, IL or NEN, Chadds Ford, PA), 10% regular DME and 10% FBS, and cultured for 48 h. Confluent monolayers were labeled when they were 2 days post-confluent and cultured for 48 h in radiolabeling medium. \(^{35}\)S-Hydroxymyidine was incorporated into DNA by labeling cultures with 2 μCi/ml \(^{3}H\)hydroxymidine in DME/10% FBS for 24-48 h.

**Whole cell lysate preparation**

BAEC whole cell (WC) lysates were prepared by washing 3- to 5-day-post-confluent monolayers with 37°C HBSS (containing Ca\(^{2+}\) and Mg\(^{2+}\)). Serum proteases were inactivated by a 10 min incubation in 4 mM diisopropylfluorophosphate (DFP) in HBSS at room temperature. Monolayers were washed twice with 4°C HBSS and the cells solubilized with 2% SDS in lysis buffer (LB: 2.5 mM imidazole, pH 7.5, containing a panel of protease inhibitors: 10 μg/ml pepstatin, 10 μg/ml leupeptin, 50 μM 1,10-phenanthroline, 5 μg/ml E64 and 2 mM EDTA). Solubilized cells were then scraped off the dish using a rubber policeman, placed into a 1.5 ml microfuge tube, sonicated briefly on ice at setting no. 5 (Branson Sonifer-Cell Disrupter 185, Danbury, CT), heated at 80°C for 15 min and stored at -20°C if not used immediately.

**Isolation of AP and BL membrane domains from tissue culture dishes**

Apical membrane (AP), basolateral membrane (BL) and internal membrane (IM) domains were isolated using a modification of the cationic colloidal silica microbead membrane-isolation procedure developed by Mason and Jacobson (1985) for HeLa cells. For higher yields of membrane during isolation procedures, glass surfaces were siliconized (Sigma Cote) and plastic surfaces were coated with Hydrion polymer (Hydrion Labs, New Brunswick, NJ) prepared as a 1.2% stock solution in 100% ethanol. Endothelial cells, confluent no longer than 5 days in 35 mm tissue culture dishes (Nunc or Costar) were washed twice at 4°C HBSS. Soluble proteases were inactivated by a 10 min incubation in 4 mM DFP in HBSS at room temperature. From this point on, all solutions and cells are maintained at 4°C. Monolayers were washed twice with HBSS and washed again in coating buffer (CB) (135 mM NaCl, 20 mM 2-(N-morpholino)ethanesulfonic acid, 1 mM Mg\(^{2+}\), 0.5 mM Ca\(^{2+}\), pH 5.5). Cells were coated with a 1% (w/v) cationic colloidal silica solution in CB. (Colloidal silica was prepared using the method of Chany and Jacobson (1983) and may be obtained from the corresponding author.) Monolayers were washed with CB then coated using a 1 mg/ml polyacrylic acid solution in CB at pH 5.0. Cells were washed again with coating buffer then washed quickly in domain lysis buffer (DLB: 2.5 mM imidazole, pH 7.5, containing 1 mM Mg\(^{2+}\) and 0.5 mM Ca\(^{2+}\) and a panel of protease inhibitors: 10 μg/ml pepstatin, 10 μg/ml leupeptin, 50 μM 1,10-phenanthroline and 5 μg/ml E64. 1,10-phenanthroline was used instead of EDTA to inhibit metalloproteases, since Ca\(^{2+}\) and Mg\(^{2+}\) are necessary to maintain cell-cell and cell-substratum contacts. Monolayers were reflexed with DLB and allowed to sit on ice for 15-30 min. Using a 5 ml syringe fitted with a shortened, flattened, blunt 18 gauge needle, shear forces were applied to the monolayer by squirting the cells with DLB at about a 45° angle. The progress of cell lysis was observed continuously under the light microscope. Lysis was determined to be complete if over 90% of the cells on the dish were lysed. The DLB-lysate solution was saved as this contained AP, IM, nuclear membrane, as well as soluble cytoplasmic fractions, of the cell. The BL membrane domains remained on the dish and were washed twice with DLB. Washes were also saved and added to the original lysate solution. The BL domain was then solubilized using about 200 μl 2% SDS in LB per 35 mm dish and treated identically to the WC solubilization procedure. BL membrane domains, although difficult to detect, were identifiable under microscopy when stained with various agents to facilitate viewing. For staining, BL membrane domains were fixed with 3% paraformaldehyde (Tousimis, Rockville, MD) in PBS, pH 7.0, containing 1 mM Mg\(^{2+}\) and 0.5 mM Ca\(^{2+}\) for 10 min. Membranes were then stained with a Coomassie Blue solution (0.1% Coomassie Blue R-250 in 10% methanol and 10% acetic acid) for 15 min and destained using 2-3 five-min washes of a 10% methanol, 10% acetic acid solution.

The lysate solution was fractionated first by a 10 min low-speed (900 g) spin to sediment nuclei and dense silica-coated AP membrane sheets. The AP membrane domains were further purified by sedimenting the low-speed pellet through a cushion of 70% (w/v) metrizamide dissolved in LB at 28,000 g for 30 min. The best results were obtained when the pellet was uniformly resuspended by brief sonication (1 second at setting...
no. 2 on a Bronson Sonifier 185 with a microprobe) and sedimented using a swinging bucket rotor (Beckman SW60Ti). Silica-coated AP membrane sheets having a density of >1.5 g/ml are considerably denser than nuclei (<1.3 g/ml) and readily sediment through the metrizamide (d=1.38 g/ml) leaving nuclei and other contaminating internal membranes at the metrizamide/hypotonic buffer interface. The IM no. 2 membrane fraction was recovered from this fraction by collecting the hypotonic solution and the material at the metrizamide interface, diluting it 2-3 times its volume with LB then pelleting the membranes for 30 min at 48,000 g. IM no. 1 is recovered from the original low-speed supernatant by centrifuging the solution for 45 min at 48,000 g. The procedure is represented schematically in Figure 1.

AP membrane proteins are recovered from the silica pellet by first washing the pellet several times in LB in a microfuge tube. The AP domains were then solubilized in 2% SDS in LB, sonicated briefly to obtain uniformity, and heated at 80°C for 15 min. Silica was then removed from the solution by sedimentation using a microfuge (14,000 g) for 10 min. The supernatant contains AP proteins and was stored at −20°C if not used immediately.

Isolation of AP and BL membrane domains from microcarrier beads

AP and BL membrane domains were also isolated from BAEc monolayers grown on Cytodex 3 (Pharmacia, Piscataway, NJ) or polystyrene microcarriers whose surface was covalently coated with gelatin (Jacobson and Ryan, 1982). Cells were grown to confluence on microcarrier beads in either roller bottles or tissue culture flasks with or without a medium as a buffer and alkalization due to CO2 loss. The roller bottle was then sealed to maintain the proper pH of the medium was gassed with 5% CO2 until a red-orange color developed, indicating that pH 7.2-7.4 was obtained. The coating of Hydron. Hydron-coating prohibits the attachment either roller bottles or tissue culture flasks with or without a medium as a buffer and alkalization due to CO2 loss was avoided. Microcarrier beads containing confluent monolayers were washed twice with 37°C HBSS. Washing was accomplished by simply suspending the beads in solution and allowing them to settle at 1 g. This prevented premature monolayer disruption that occurs with rougher treatments such as centrifugation. From this point on, all solutions were maintained at 4°C. Beads were washed once with HBSS, serine proteases were inactivated with DFP, beads were washed in HBSS and washed again in CB. Cells were coated with a 1% (w/v) cationic colloidal silica solution in CB. Microcarriers were washed with coating buffer then coated using a 1 mg/ml polyacrylic acid in CB at pH 5.0. Cells were washed again with CB then washed quickly in DLB. After resuspension in DLB, the microcarriers were set in ice for 30 min. Lysis was performed by gently vortexing the beads. This procedure makes the beads crash together, sheering off the AP membrane, leaving the BL domain on the microcarrier. The extent of the disruption was monitored under the light microscope. BL membranes on the microcarriers were washed 3 times in DLB and prepared for scanning electron microscopy (see below) or solubilized in 2% SDS in DLB. AP membranes were treated exactly as in the dish procedure for solubilization and scanning electron microscopy.

One-dimensional gel electrophoresis and immunoblotting

Proteins from isolated membrane domains were resolved by using 8% one-dimensional discontinuous sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE: 8% acrylamide, 0.72% bis) as described by Laemmli (1970). Polyacrylamide (Mw = 500,000) was added to each gel to a final concentration of 0.17% to give added strength to the gel. Proteins obtained from WC lysates or isolated membrane domains were resolved electrophoretically on 8% SDS-PAGE mini-slab gels and transferred to nitrocellulose (0.22 μm pore size; Schleicher and Schuell, Keene, NH) as described (Towbin et al. 1979). The nitrocellulose was blocked with 5% nonfat powdered milk (Carnation) in Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) and subsequently probed with monoclonal antibodies to angiotensin-converting enzyme (ACE, clone 2C6D3CF, kind gift from Dr. Linda Slakey, University of Massachusetts, Amherst), rabbit polyclonal antibodies to collagen receptors (kindly provided by Dr. Dorothy Beacham) or monoclonal antibodies to cytochrome oxidase subunit no. 2 (clone CCO6, generous gift from Dr. Thomas Mason, University of Massachusetts, Amherst). Alkaline phosphatase or horseradish peroxidase conjugated to either goat anti-mouse or anti-rabbit IgG were used as secondary antibodies and color was developed according to the manufacturer's instructions.

Protein, chemical and enzyme assays

Protein in each SDS-solubilized sample was determined using the biuret method (Smith et al., 1985) with reagents from Pierce Chemical Co. (Rockville, IL) or Sigma. Care was taken to wash thoroughly membranes sedimented through metrizamide, since metrizamide strongly interferes with Lowry (Slayby and Farquhar, 1977) and BCA (Stolz and Jacobson, unpublished results) protein assays. Samples containing silica were solubilized by 1% SDS and heating to 80°C for 15 min, the silica was pelleted at 14,000 g in a microfuge and the supernatant assayed. Silica was quantitated on SDS-solubilized membrane domains (in this case, silica was not pelleted) using the procedure developed by Iler (1979). Briefly, a 100 μl sample was added to 400 μl of 1 M NaOH and heated at 90°C for 45 min to solubilize the colloidal silica. To this solution, 2 ml of ammonium molybdate in 0.75 M H2SO4 was added and the absorbance read at 410 nm. The ammonium molybdate was made up as a stock solution to 81 mM in 1.75 M NH4OH. One ml of this solution was diluted with 7 ml of 0.86 M H2SO4.

In order to characterize and determine enrichments of isolated membranes, several techniques for monitoring membrane marker enzymes were used. DNA was quantitated colorometrically by the diphenylamine method (Schneider, 1957). DNA was also determined by using a [3H]thymidine/5'-methylthymidine double labeling method and [3H]thymidine/protein determinations. Alkaline phosphatase activity was determined using the methods developed by Green and Newell (1974) and modified by Shiozawa et al. (1987). Endoplasmic reticulum marker glucose 6-phosphatase activity was determined using the method of Aronson and Touster (1971). Thiamine pyrophosphatase, a Golgi apparatus marker, was determined using the method of Melodosi et al. (1971). Ouabain-sensitive Na+/K+-ATPase activity was determined from freshly isolated membrane domains (not previously frozen) using the method of Haggerty et al. (1978). Lysosomal activity was determined using the β-hexosaminidase assay modified from Hubbard and Cohn (1975) and Dr. Jack H. Nordin (personal communication). Briefly, 5-20 μl of isolated membrane domains or WC lysates were added to 8 mM MES, pH 5.3, to a final volume of 100 μl. Sixty microliters of 10 mM β-N-acetyl glucosaminide p-nitrophenyl phosphate was added to each mixture to start the reaction.

Endothelial cell membrane protein polarity

41
Each sample was incubated for 60 min at 37°C with occasional agitation. The reaction was stopped by addition of 50 μL of 0.2 M Na₂CO₃ to each sample and vortexing briefly. Samples were read at 405 nm and compared to a standard curve of 0-200 nM p-nitrophenol.

For all marker enzyme assay procedures, freshly isolated membranes were not solubilized in 1% SDS but suspended in a solution of 0.25 M sucrose, 5 mM Tris-HCl, pH 8.0, before each assay procedure. All samples were stored frozen at -20°C, except where noted, prior to the assays. In order to conserve sample, total assay volumes were reduced to 200-500 μL in all assays so they could be read either in microtiter plates or in 500 μL cuvettes.

Relative specific activities for each isolated membrane domain were determined by first calculating the enrichments for each membrane domain relative to the WC lysate within a single experiment, then enrichment averages ± s.d. were calculated by pooling the enrichment data for each domain from a number of experiments.

Transmission electron microscopy
To prepare for TEM, silica-coated monolayers and isolated membrane domains were processed as described above. Coated cells and domains were then fixed in 2% paraformaldehyde (Tousimis) and 1% glutaraldehyde (Polysciences) in PBS with 0.5 mM Ca²⁺ and 1 mM Mg²⁺, pH 7.0, at 20°C for 1 h while shaking. This solution was removed, the cells were washed 3× in PBS and postfixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate, pH 7.0, while shaking. Cells were washed 3× in NANOpure water, then stained in freshly made, 0.22 μm filtered 0.1% uranyl acetate for 30 min while shaking. Cells were washed again 3× in water, then dehydrated through a series of isopropanol solutions (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% and 4× in 100%) at 10 min each step. Samples were critical point dried (Polaron, Watford, England), sputter coated (Polaron E5000), and viewed on a JEOL 25S scanning electron microscope at 15 kV.

Results
Morphological analysis
Using the procedures shown schematically in Figure 1 and delineated in Materials and methods, AP and BL membrane domains were isolated from 3- to 5-day post-confluent BAEC monolayers. Domain isolation could

![Fig. 1. General apical/basolateral membrane-domain isolation scheme for endothelial cell monolayers cultured in tissue culture dishes. Letters at each stage of the isolation procedure are also represented by a scanning electron micrograph in Fig. 3.](image-url)
be readily viewed by phase-contrast light microscopy (Fig. 2). Under normal isolation conditions, most (~90-95%) by visual inspection after Coomassie blue staining as in Fig. 2) of the BL membrane remained attached to the dish. The balance of the tissue culture dish surface was either areas where the membranes had become detached (clear areas “S” of Fig. 2C) or whole cells that had escaped lysis (dark areas “WC” of Fig. 2B,C). Occasionally (10-15% of the procedures), a monolayer would completely float off the dish as an intact sheet. This occurs when a culture has been confluent on the dish for longer than 5 days, or if “sprouters” subending the monolayer are visible within the culture. Cultures which exhibit this behavior were not used for experiments. Numerous open AP membrane sheets are also visible within the post-lysis solution (Fig. 2D), as are intact nuclei and cell debris. BL membrane domains attached to the culture dish can also be visualized by fluorescence microscopy after labeling them with a fluorescent probe that partitions into membranes (Stolz et al., 1992).

The AP and BL membrane isolation procedure is verified at higher resolution by scanning electron microscopy (SEM) (Fig. 3). The silica-polycrylate-coated cell monolayer is seen in Figure 3B and b, displays an even “pebbly” texture when compared with the uncoated monolayer (Fig. 3A and a). At greater magnification, ultrastructure associated with the isolated domains is readily apparent. The AP membranes are isolated as open sheets (Fig. 3C and c) while the BL membrane domain, still flattened, remains attached to the tissue culture dish surface (Fig. 3D and d) and many of the lateral membrane edges that delineated the cell are left intact. The fiber-like material seen attached to the cytoplasmic aspect of the isolated AP and BL membrane domains is believed to be part of the submembranous cytoskeleton (Fig. 3C,c and D,d). Separate experiments observed the binding of rhodamine-phalloidin (Mason and Jacobson, 1985) to isolated AP and BL membrane domains (data not shown), indicating that F-actin was present on the isolated membrane domains, therefore corroborating the SEM findings.

The isolation procedure could also be used with cells grown on Cytodex 3 microcarriers (Pharmacia) or polystyrene microcarriers whose surface is covalently coated with gelatin (Jacobson and Ryan, 1982). Domains were isolated in much the same way as those grown on culture dishes, except that instead of cell rupture by shear forces from a stream of buffer, the beads could be briefly, but vigorously, vortexed. The microcarrier procedure was verified by viewing the isolated domains by SEM (Fig. 4). The advantages of using microcarriers was the increased surface area afforded by the beads and the time saved by vortexing beads as opposed to squirting monolayers in dishes. This was the preferred approach when large amounts of membrane protein (>1 mg) were needed. However, for most of the studies discussed here, domain isolation from 10-20 35-mm culture dishes produced sufficient amounts of protein.

Ultrasound examination by TEM was also conducted on BAEC monolayers during the cationic silica microbead membrane-domain isolation procedure. Figure 5A shows that the pellicle of cationic colloidal silica, visible as electron-dense beads having an average diameter of 20-50 nm, forms an even silica coating, closely apposed to the AP PM surface. The silica does not penetrate into the plasmalemma vesicles or through the intercellular junctional complexes located at areas of cell-cell contact, and is therefore not found associated with the BL PM surface. The isolated, density gradient-purified, colloidal silica-coated AP membrane pellet was also examined ultrastructurally. At low magnification, a cross-section of the silica-membrane pellicle appears to contain long sections of linear arrays of silica-associated PM (Fig. 5B). At higher magnification (Fig. 5C), the linear arrays of silica are still closely apposed to the AP membrane surface and many of the plasmalemma vesicles also remain intact and connected to the isolated PM.

Biochemical analysis

The relative specific activities (enrichment data) for known marker enzymes of intracellular organelles, as well as known domain-specific markers (ACE and silica for the AP domain and Na+K+-ATPase, and collagen receptors for the BL domain) were determined for each isolated membrane fraction. Table 1 shows that the cationic silica membrane-domain isolation procedure enriches silica in the AP PM domain 4- to 9.6-fold over WC lysates. However, it also shows that silica is often slightly enriched (0.7- to 2.1-fold) in the BL domain. We believe this is artifactual in that when BAEC monolayers, grown in 35 mm tissue culture dishes, are coated with the silica pellicle, silica also adheres to the vertical sides of the dish that do not contain cells but do contain adsorbed serum proteins. When BL membrane domains are solubilized, scraped and collected from the dish, a portion of this residual silica is included in the BL membrane domain preparation. As shown in Figure 5A, silica is not seen on the BL domain when thin sections of confluent monolayers coated with the silica-polycrylate pellicle are examined by TEM. The intercellular junctions exclude the silica microbeads from the BL membrane surface.

ACE has been shown to be an endothelial cell luminal domain marker in vivo (Gumkowski et al. 1987; Laliberte et al. 1987; Ryan et al. 1976a,b), an AP domain component in vitro (Nakache et al. 1986; Ryan et al. 1976a,b) and was determined to be 5- to 15-fold enriched in the AP membrane domain over WC lysates of cultured BAECs when isolated membrane domains were immunoblotted with monoclonal antibodies to ACE. ACE was not detected in immunoblots of isolated BL membrane domains. Shown to be an endothelial cell BL marker in vivo (Betz et al. 1980; Vorbrot, 1988), ouabain-sensitive Na+K+-ATPase (Haggerty et al. 1978) was enriched 3.55- to 3.67-fold in the BL membrane domain over WC lysates and de-enriched 0.10- to 0.36-fold in AP membrane fractions. The ouabain-sensitive Na+/K+-ATPase activity in the
Fig. 2. Light micrograph representing isolation of membrane domains from BAEC monolayers. (A) A confluent monolayer fixed with 3% paraformaldehyde and stained with 0.1% Coomassie blue (described in Materials and methods) to facilitate viewing of isolated BL PM domains. (B) The isolated BL membrane aspect of the monolayer following domain isolation. The BL membrane domains have been fixed in 3% paraformaldehyde in PBS and stained with 0.1% Coomassie blue. Arrowheads indicate nuclei (N) of lysed cells and open arrows indicate the lateral aspect of the BL membrane domain that forms an outline “footprint” of the cells following domain isolation. WC indicates the unfixed whole cells still present in the preparation. (C) Another area of the same culture dish following domain isolation in which incomplete isolation (presence of WC), as well as complete stripping of the basolateral domain (S) is evident in the preparation. This panel is designed to show that there is indeed basolateral membrane domain (BL) left adhering to the dish and not simply stripped culture dish surface (S). (D) A field of typical cell lysate which contains unidentified cell debris, nuclei (N) as well as large open sheets of AP membrane that have been detached from the monolayer (arrows) following the silica membrane domain isolation procedure. The subcellular fractions in this panel have not been fixed or stained. Bar, 100 μm.
isolated BL fraction represents 9-12% of the total ATPase activity in this fraction. This is lower than the 29% reported by Bonting et al. (1961) for cat aortas. If the difference is not due to species specificity or the fact that whole aortas were used and not the isolated endothelium, it may be that the loss of activity is a result of culturing conditions, since others have reported that enzyme activities of endothelial cells can diminish with time in culture (Gordon et al. 1991). It may also be due to a partial inaccessibility of the substrate to interact with the enzyme in the isolated BL fractions. It was previously shown that the accessibility of proteins on the silica-coated surface of the PM was reduced to degradation by proteases activity and labeling by lactoperoxidase-catalyzed iodination (Chaney and Jacobson, 1983). However, it is this inaccessibility to such protein-modifying reagents that permits one to determine whether a protein has domains exposed on one or both sides of the PM, by labeling the proteins either before silica-coating the cell or after membrane isolation.

Receptors to the extracellular matrix protein collagen (CR), a BL membrane domain-enriched receptor (Lu et al., 1992), was found to be enriched in BL membrane domains when immunoblotted with rabbit anti-HeLa cell CR serum. Western blots using polyclonal antibody preparations displayed uneven distribution of CRs between the AP and the BL domains: the CRs isolating
Fig. 4. Scanning electron micrographs of various stages of the apical/basolateral PM-domain isolation technique accomplished using BAEC monolayers cultured on gelatin cross-linked to the surface of polystyrene microcarriers. (A) Plain gelatin-coated microcarrier. (B) Microcarrier covered with a confluent monolayer of BAECs. Spaces between cells are due to cell shrinkage during SEM preparatory dehydration. (C) Cells covered with a silica and polyacrylate pellicle. (D) Microcarrier in mid-stage of apical/basolateral PM-domain separation accomplished by hypotonically swelling cells then vigorously vortexing the beads. Traces of apical membranes are apparent as sheets attached to the microcarrier. Basolateral membrane domains are visible as is putative cytoskeletal “fibrous” material covering the surface of the bead. (E) Higher magnification of a basolateral domain remaining on the microcarrier surface. (F) Magnification of an isolated apical domain sheet. ex, exterior of the apical membrane sheet coated with silica; in, interior, cytoplasmic face of the apical membrane sheet. Bars: A-D, 100 μm; E and F, 10 μm.

with the BL membrane domain were 10- to 40-fold enriched over WC lysates and de-enriched 0.35- to 0.55-fold in the AP membrane domain (Table 1).

The results from assays monitoring activities of intracellular organelles showed minimal contamination of the intracellular organelle markers in the isolated AP and BL membrane domains (Table 1). Relative enrichments of specific intracellular organelle markers were consistently found in the high-speed pellet IM no. 1 derived from the supernatant of the first low-speed pellet (see Fig. 1 flow chart, Materials and methods). The IM no. 2 pellet (material not dense enough to sediment through the 70% metrizamide cushion) contains AP membrane connected to other organelles and cytoskeletal proteins (B. S. Jacobson, J. E. Schnitzer and G. E. Palade, personal communication) and are less dense than the 1.38 g/ml necessary to sediment through the metrizamide (hence the enrichment of silica in this fraction). It should also be noted that that the interfacial tension between the buffer and the dense metrizamide solution would tend to prevent smaller fragments of silica-coated AP membrane domains from sedimenting through the cushion.

Alkaline phosphatase (Shiozawa et al. 1987), a marker of total membrane, was enriched in all membrane fractions as reported previously (Betz et al. 1980; Vorbroidt, 1988). Glucose 6-phosphatase, an endoplasmic reticulum marker (Aronson and Touster, 1974), was de-enriched in both the AP and BL membrane domain preparations. Thiamine pyrophosphatase (TPPase), a Golgi apparatus membrane marker (Meldolesi et al. 1971), was de-enriched in BL domains but very slightly de-enriched to slightly enriched in AP fractions. It has been shown previously that TPPase
Fig. 5. Ultrastructural examination of confluent monolayers and isolated apical membrane sheets coated with the silica-polyacrylate pellicle. (A) The silica pellicle (Si, curved arrow) is confined to the apical (AP) membrane surface of BAEC monolayers in vitro. The silica does not penetrate to the basolateral (BL) membrane domain via the intercellular tight-junctional structures (arrows). The silica does not enter plasmalemma vesicles (arrowheads) that are open the AP PM domain. Bar, 0.5 μm. (B) Low magnification of a cross-section through the isolated AP membrane pellet. The AP membranes are isolated as long, continuous sheets of silica attached to the membrane. Bar, 1 μm. (C) At higher magnification, the isolated AP membrane domains show close apposition of membranes to the silica microbeads (Si, curved arrow), and the plasmalemma vesicles (arrowheads) are also isolated intact with the AP membrane domain. Bar, 0.5 μm.

Activity is detectable on the luminal surface of brain endothelium in vivo using cytochemical enzyme-detection procedures of tissue sections (Vorbrodt, 1988) and this phenomenon also appears to be true for AP domain of BAECs in vitro. Lysosomal activities as reflected by β-hexaminidase assays (Hubbard and Cohn, 1975) indicate minimal lysosomal contamination in either AP or BL membrane domain fraction. Immunoblots of membrane fractions with monoclonal antibodies to the mitochondrial enzyme cytochrome oxidase, indicate a signal only in the IM no. 1 fraction (data not shown). Another interesting finding was that a slight enrichment of DNA was found in isolated AP membrane domains. The nature of this enrichment was tested using a double-labeling procedure and [3H]thymidine was found to be 14-fold enriched in the residual silica pellet after SDS solubilization of the AP membrane proteins. This indicates that, during the isolation procedure,
DNA (which is uniformly very negatively charged) is released from disrupted nuclei and may tightly associate with the cationic silica. The DNA appears to be very tightly associated with the silica, and 2% SDS, heating to 80°C, and vigorous sonication does not release the DNA (which is uniformly very negatively charged) is assigned to the IM no. 1 fraction.

The yield of isolated AP and BL membrane fractions using the cationic silica microbead isolation protocol is much higher and less cumbersome than those reported for endothelial cells using other procedures (Jaffe et al., 1987). The yield of silica, representing the AP membrane surface was 43-52% and the yield of Na⁺/K⁺-ATPase, representing the BL surface, was 75-81%. The lower yield of AP membrane is most likely due to the greater number of steps used to isolate the AP membrane compared to the BL membrane, and this subsequently results in the higher relative enrichment of AP membrane (4- to 9.6-fold) versus BL membrane (3.56- to 3.67-fold).

Previous results obtained from membrane domains isolated from HeLa cells lactoperoxidase-radio-iodinated in suspension indicated that there was ~80% yield of each membrane fraction using the silica microbead technique (Mason and Jacobson, 1985). However, the metrizamide cushion, which tends to reduce the yield, was not used in these experiments. Labeling suspensions of BAECs with sulfo-/V-hydroxy-succinimido-biotin, followed by plating and membrane domain isolation also indicated about an 80% yield for isolated AP and BL membranes when the biotin was quantitated with alkaline phosphatase-conjugated avidiin (L. Baldwin and B. S. Jacobson, unpublished observations). Likewise, preliminary experiments using bead-immobilized lactoperoxidase AP-domain-specific radio-iodination of confluent BAEC monolayers (Muller and Gimbrone, 1986), followed by colloidal silica AP and BL membrane-domain isolation, suggested an 85% (n=14) recovery of all membrane domains (B. Conradt and B. S. Jacobson, unpublished observations). However, different 1-D gel radio-iodinated protein patterns of the AP and BL PM domains resulted when 5-day confluent monolayers were labeled in this manner. This suggested the radiolabeling was not restricted to the AP membrane surface but penetrates confluent monolayers to label some BL PM proteins (B. Conradt and B. S. Jacobson, unpublished observations). Biotin derivatives were also often found to penetrate the junctions of confluent BAEC monolayers (B. Conradt and B. S. Jacobson, unpublished observations). We concluded that domain-specific labeling was unreliable and was therefore not used to study transcellular membrane protein polarity of BAEC monolayers (see also Discussion).

### Table 1. Relative specific activities of enzymes, proteins and specific marker molecules in isolated membrane domains

<table>
<thead>
<tr>
<th>Assay</th>
<th>AP</th>
<th>BL</th>
<th>IM no. 1</th>
<th>IM no. 2</th>
<th>Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline phosphatase (total PM)</td>
<td>2.30±0.43</td>
<td>1.35±0.43</td>
<td>3.01±2.38</td>
<td>1.11±0.60</td>
<td>ND</td>
</tr>
<tr>
<td>Silica enrichment (apical membrane)</td>
<td>6.77±2.79</td>
<td>1.41±0.74</td>
<td>0.59±0.19</td>
<td>1.58±0.53</td>
<td>ND</td>
</tr>
<tr>
<td>Angiotensin-converting enzyme* (apical membrane)</td>
<td>5-15</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase (basolateral membrane)</td>
<td>0.23±0.13</td>
<td>3.61±0.06</td>
<td>1.82±0.79</td>
<td>0.49±0.28</td>
<td>ND</td>
</tr>
<tr>
<td>Collagen receptors* (basolateral membrane)</td>
<td>0.35-0.55</td>
<td>10-40</td>
<td>ND</td>
<td>3-6</td>
<td>–</td>
</tr>
<tr>
<td>Glucose 6-phosphatase (endoplasmic reticulum)</td>
<td>0.58±0.22</td>
<td>0.62±0.29</td>
<td>3.61±1.48</td>
<td>0.62±0.48</td>
<td>ND</td>
</tr>
<tr>
<td>Thiamine pyrophosphatase (Golgi apparatus)</td>
<td>1.05±0.24</td>
<td>0.68±0.25</td>
<td>2.45±0.85</td>
<td>0.66±0.32</td>
<td>ND</td>
</tr>
<tr>
<td>β-Hexaminidase (lysosomes)</td>
<td>0.02±0.00</td>
<td>0.36±0.20</td>
<td>1.53±0.66</td>
<td>0.51±0.30</td>
<td>ND</td>
</tr>
<tr>
<td>Cytochrome c oxidase† (mitochondria)</td>
<td>ND</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
<td>–</td>
</tr>
<tr>
<td>DNA (nucleus/diphenylamine assay)</td>
<td>1.18±0.74</td>
<td>0.44±0.14</td>
<td>1.67±0.54</td>
<td>0.67±0.47</td>
<td>–</td>
</tr>
</tbody>
</table>

* Determined by immunoblotting and enrichment values assigned by videodensitometry.
† No measurable cytochrome oxidase signal was found in the WC lysate fraction; therefore, relative enrichment value could not be assigned to the IM no. 1 fraction.

**Discussion**

In this report we evaluate the cationic colloidal silica...
microbead membrane-domain isolation technique for the examination of AP and BL membrane protein asymmetry in BAEC monolayers. This proved to be a rapid technique for obtaining high yields of 3- to 9-fold enriched AP and BL membrane domains from BAEC monolayers grown on either tissue culture dishes or microcarrier supports. Others have approached the endothelial cell polarity question using techniques that we have found useful, but unfortunately limited. Jaffe et al. (1987) employed a membrane fractionation procedure followed by a sucrose density step gradient in order to isolate two distinct membrane fractions from BAEC monolayer cultures. One fraction, highly enriched in both ACE and alkaline phosphatase activity was believed to be of AP origin, while a heavier fraction, 8-fold enriched in Na\(^+\)/K\(^+\)-ATPase was postulated to be of BL origin. These isolated membrane fractions were immunoblotted with a monoclonal antibody against BAECs, which was found to react with a 130 kDa protein located primarily in the ACE enriched fraction, or, when examined by immunofluorescence microscopy, localized to the luminal domain of bovine tissues. While these data confirm the existence of defined membrane domains within a monolayer, it would be difficult to study the involvement of other factors influencing polarity, such as cytoskeletal interactions or the restriction of AP/BL lateral diffusion by junctional complexes, using this technique. Very low yields of purified membrane domains and the need for huge amounts of cells make this procedure quite cumbersome as well as limited for such studies.

Novel procedures describing domain-specific radioiodination of endothelial cell monolayers using radioiodination catalysts on microbead supports have been published by Ghinea and colleagues (1989) and by Muller and Gimbrone (1986). The latter authors developed a labeling procedure whereby AP and BL membrane domain proteins could be selectively radioiodinated. By using lactoperoxidase (LPO) covalently coupled to 0.7 μm latex beads, iodination of proteins in the AP domain of confluent monolayers was achieved by addition of glucose oxidase to generate H\(_2\)O\(_2\). Labeling the BL membrane domain was attained by first lifting the confluent monolayer off the culture dish via the application of a polylysine-coated coverslip to the AP domain, then proceeding with the iodination of the newly exposed BL domain proteins with the LPO-latex beads. The asymmetric distribution of radioiodlated proteins was then determined by collecting only those proteins that had partitioned into a Triton X-114 detergent-rich phase.

We encountered three major problems with this procedure. First, Triton X-114, a non-ionic detergent similar to Triton X-100, has been found not to extract all proteins noncovalently associated with the cytoskeleton (Lu et al. 1989). Therefore, PM proteins, that may be highly glycosylated or associated with the cytoskeleton, probably would not partition into the detergent-enriched solution under the conditions used in the experiments (Clemetson et al. 1984; Maher and Singer, 1985; Pryde, 1986; Pryde and Phillips, 1986). Therefore, it appears that the investigators looked at only a small, specific subset of asymmetrically distributed labeled PM proteins (i.e. those that are not interacting with the cytoskeleton), since it is now known that some proteins segregated to either AP or BL domains are maintained in their positions by interaction with the submembranous cytoskeleton (Nelson, 1989). The above indicates that if a protein were not associated with the cytoskeleton in one domain but were associated with it in the opposite domain, the Triton X-114 procedure used by Muller and Gimbrone (1986) would result in an incorrect assignment of the asymmetric distribution of the protein. Another shortcoming in the protocol involves the removal of the confluent monolayer by physically pulling it off the culture dish. It is very likely that conditions as harsh as these will leave behind patches of membrane and protein that comprise the numerous focal contact (adhesion) sites of the cells that tenaciously anchor the cell to the substratum (Cathcart and Culp, 1979; Culp et al. 1980; McNeil et al. 1984). By leaving these “pinched-off’ vesiculated domains on the dish, a subset of BL membranes might not be represented in the labeled BL fraction. Thirdly, the most serious drawback that we encountered, was that the radio-labeling of surface proteins was not confined to the AP membrane surface. When confluent BAEC monolayers were labeled from the AP side as previously described (Muller and Gimbrone, 1986), then membrane domains isolated by the cationic colloidal silica membrane-domain isolation procedure and proteins subsequently resolved by SDS-PAGE autoradiography, very different radio-iodinated protein patterns representative of the isolated AP and BL membrane domains resulted (B. Conradt and B. S. Jacobson, data not shown). This suggested that the labeling procedure was not restricted to the AP membrane surface, and BL membranes were also getting labeled. This is not surprising, since it is now known that radio-iodine radicals from LPO-H\(_2\)O\(_2\) catalysis could easily penetrate a cell membrane and label any protein indiscriminately (Huber et al. 1989). Thus, while AP/BL protein asymmetry was observed by the procedures described by Muller and Gimbrone (1986), it is questionable whether the asymmetry was real or a consequence of the techniques used.

The AP PM domain, when isolated by the procedure described here, is coated with silica (Fig. 5A), and remains as open membrane sheets possessing one silica-coated side (external leaflet) and one cytoplasmic side (internal leaflet), which can be easily discerned using SEM (Figs 3 and 4). Upon greater magnification, a submembranous cytoskeletal network is apparent on the cytoplasmic leaflet of the isolated membrane domains (Figs 3c,D,d, 4E and F). Unfortunately, the silica pellicle visually obscures any membrane ultrastructure facing the extracellular leaflet. The procedure also maintains a membrane protein’s temporal and spatial locations at the exact moment silica coats the membrane on through to the completion of membrane isolation. It has been shown, using the cellular slime mold Dictyostelium discoidium, that lectin-induced
patching and capping of cell surface glycoproteins retain their degree of aggregation and resist lateral diffusion once the PM has been silica-coated and isolated (Patton et al., 1989). The silica-coated AP membrane also retains many of its associated plasmalemma vesicles (Fig. 5C), specialized transcellular transport structures characteristic of endothelial cells (Simionescu and Simionescu, 1988). It has also been shown to retain association with other internal structures like the budding of enveloped viruses (Samby and Rodriguez-Boulan, 1988). Likewise, the BL domain retains its ultrastructure, since the membranes are still attached to the substratum and are not released from the dish surface or vesiculate after the AP domain is removed. This could be due to the fact that the cytoplasmic leaflet of the BL membrane domain, like the isolated AP membrane domain, shows that a submembranous cytoskeleton remains intact (Figs 3 and 4), presumably sandwiching the lipid membrane bilayer between cytoskeleton and extracellular matrix via integral PM/protein interaction.

The sum of the data presented above strongly indicates we have successfully isolated two separate, distinct PM fractions in high yield from endothelial cells in vitro and the enzyme marker analysis correlates positively with what is known about AP and BL PM domains from endothelial cells in vivo. We have expanded the use of this procedure to examine the role of the submembranous cytoskeleton and intracellular junctional complexes in the maintenance of PM protein asymmetry in BAEC monolayers in vitro (Stolz et al., 1992).

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