Vascular Proteomics and Subtractive Antibody Expression Cloning*

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The cloning of genes expressing proteins that are differentially expressed in the organ microvasculature has the potential to address a variety of problems ranging from the analysis of disease pathogenesis to drug targeting for particular tissues. This study describes a methodology designed to analyze differential protein expression in the brain microvasculature. The method can be applied to other organs and is particularly suited to the cloning of cDNAs encoding membrane proteins. The technology merges a tissue-specific polyclonal antiserum with a cDNA library expression cloning system. The tissue-specific antiserum is subtracted with protein extracts from control tissues to remove those antibodies that recognize common antigenic proteins. Then, the depleted antiserum is used to expression clone tissue-specific proteins from a cDNA library expressed in mammalian cells. The methodology was evaluated with a rabbit polyclonal antiserum prepared against purified bovine brain capillaries. The antiserum was absorbed with acetone powders of liver and kidney and then used to screen a bovine brain capillary cDNA library in COS cells. The initial clone detected with this expression methodology was the Lutheran membrane glycoprotein, which is specifically expressed at the brain microvasculature compared with liver and kidney tissues. This subtractive expression cloning methodology provides a new approach to "vascular proteomics" and to the detection of proteins specifically expressed at the microvasculature, including membrane proteins. Molecular & Cellular Proteomics 1:75–82, 2002.

The identification of proteins that are differentially expressed between tissues is critical to the illumination of mechanisms involved in both tissue development and tissue differentiation. Elucidation of differentially expressed proteins also has the potential to identify proteins that participate in the pathogenesis of diseases such as angiogenesis, aberrant proliferation, and altered cellular behavior. In addition, transport systems that are unique to a certain tissue or cell type could lead to novel targets for drug transport to specific tissue sites. Tissue-specific channels, endocytosing systems, and cellular receptors could function as such targets and mediate cell-specific and organ-specific uptake.

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Tissue-specific gene expression can be evaluated with functional genomics that focus on the detection of tissue-specific mRNAs. However, the correlation between the protein and mRNA contents of the cell is not necessarily linear, because these levels are dynamic species regulated by distinct factors (1). An example of this regulation can be seen in the 3'-untranslated regions of mRNA where regulatory proteins can bind and play a role in determining the post-transcriptional regulation of gene expression (2). Technologies used to analyze the protein content of cells include traditional expression cloning methods where a known target is extracted from a cDNA library (3, 4) with a specific antibody. However, without a priori knowledge of a specific target, and without the availability of a specific antibody directed against the protein target, this method is limited. Other large scale differential protein expression analyses include 2-dimensional gel electrophoresis (5, 6) and subtractive expression cloning using phage display (7). Although 2-dimensional gels can yield a panel of differentially expressed proteins, it is difficult to analyze membrane proteins because of solubility constraints (5). The method of coupling phage display of a cDNA library and a tissue-specific polyclonal antiserum can identify differentially expressed proteins (7) but is potentially limited by host biology and the rudimentary prokaryotic post-translational machinery (8). This may be especially exacerbated in the case of library constituents that encode mammalian membrane proteins, which require proper folding to be antigenic on the surface of the phage particle (8, 9).

In the present study, a robust proteomics technology was developed that allows the identification of tissue-specific proteins, including membrane proteins. The methodology is initially applied to the brain microvasculature, which forms the blood-brain barrier (BBB)1 in vivo. A polyclonal antiserum against bovine brain microvessel endothelial proteins was depleted with protein preparations from liver and kidney tissues, which removes the antigenicity to commonly expressed proteins (10). This depleted or subtracted antiserum was then used to screen a bovine brain capillary cDNA library in COS cells to identify proteins that were differentially expressed at the brain microvasculature, as compared with liver and kidney. The technique was vali-

1 The abbreviations used are: BBB, blood-brain barrier; X-gal, 5-bromo,4-chloro,3-indoyl β-D-galactoside; PBS, phosphate-buffered saline; PBST, PBS containing 0.05% Tween 20; B-CAM, B-cell adhesion molecule.
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dated with the cloning of the Lutheran membrane glycoprotein, which is a brain vascular-enriched protein. The method has the potential to identify molecular transporters for non-invasive drug delivery to the brain and can be applied to many other tissue types and systems.

EXPERIMENTAL PROCEDURES

Bovine Brain Capillary cDNA Library Construction—Bovine brain microvessels were isolated from total bovine brain homogenates, and the brain capillary-derived mRNA was purified as described previously (11). The integrity of the capillary mRNA was confirmed by actin Northern blot analysis (11). Full-length cDNA inserts were created using the Superscript system (Invitrogen). The mRNA was reverse-transcribed using the NRT oligo(dT) primer adaptor, and the 5′ ends of the cDNA inserts were ligated to BstXI adapters (Invitrogen) to allow directional cloning into the pcDNA1.1 mammalian expression vector (Invitrogen). The ligated cDNA library was transformed into MC1061/P3 bacteria (Invitrogen) using electroporation, and insert-containing clones were selected using tetracycline (10 μg/ml) and ampicillin (50 μg/ml)-containing medium. The bovine cDNA library consisted of antibiotic-resistant bacterial clones arising from between 40 and 5 × 10⁶ electroporation reactions and yielded a total library size of 5 × 10⁶ clones. The library pools were analyzed with BamHI-Xhol double restriction digest to release the cDNA inserts, and the size distribution was extended from 0.6 to 12 kb.

Cell Culture and Transfection—COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) containing a high level of glucose (4.5 g/liter) buffered by sodium bicarbonate (2.0 g/liter) and HEPES (7.2 g/liter) to pH 7.2 and supplemented with 10% fetal bovine serum (Invitrogen). Cells were plated 24 h prior to transfection at a density (8 × 10⁴ cells/cm²) providing a confluent monolayer of cells for the day of transfection.

To optimize transfection efficiency, a plasmid containing the gene for Escherichia coli β-galactosidase under control of the SV40 promoter (Promega, Madison, WI) was used as an in situ diagnostic for the identification of transformed cells. LipofectAMINE was used in conjunction with Plus reagent (Invitrogen) following the manufacturer’s protocol, and the conditions were optimized to yield between 40 and 50% β-galactosidase-positive COS-1 cells. The optimized conditions were as follows: COS-1 cell monolayer, 2 μl/cm² Plus reagent, 1.5 μl/cm² LipofectAMINE, 0.2 μg/cm² column-desalted maxiprep plasmid DNA (Qiagen, Valencia, CA). Column desalting of maxiprep DNA was critical for successful transfection with LipofectAMINE, and each of the additional parameters was optimized over a broad working range of concentration and cell density.

DEAE-dextran transfection conditions were also optimized using the β-galactosidase gene as a probe. The transfection mixture was optimized and consisted of the following: 2 μg/ml plasmid DNA, 0.1 mM chloroquine, 400 μg/ml DEAE-dextran (molecular mass = 5 × 10⁴ Da), in COS-1 growth medium without sodium bicarbonate, antibiotics, or fetal bovine serum (DMEM-H). Prior to incubation with the transfection mixture, the cell monolayers were washed with DMEM-H. Enough transfection mixture (DNA, DEAE-dextran, and chloroquine) was added to the plates to adequately cover the cell layer. The cells were incubated at 37 °C for 4 h, and the solution was redispersed every 30 min by gently rocking the plates from side to side. The transfection mixture was removed, and the cell layer was washed with DMEM-H. The cells were shocked with 37 °C preheated 10% Me₂SO in 0.01 M PBS, pH 7.2, for 2 min. The Me₂SO solution was removed, and the cells were washed twice with DMEM-H followed by addition of complete serum without antibiotics. The presence of chloroquine and the use of Me₂SO shock were required for maximum efficiency, and this method was not nearly as sensitive to a DNA desalting step as when LipofectAMINE was used. Cell density and DNA concentration were optimized over a broad range. This optimized transfection protocol achieved transfection efficiencies from 10 to 20%.

Analysis for β-galactosidase activity was performed by fixing transfected cell monolayers with 0.5% glutaraldehyde in 0.01 M phosphate-buffered water, washing with phosphate-buffered water, and incubating with 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal) substrate solution (0.01 mM NaHPO₄, pH 7.0, 0.15 mM NaCl, 35 mM K₂Fe(CN)₆, 35 mM K₃Fe(CN)₆, 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 1 mg/ml X-gal) at 37 °C overnight for blue color development. It was determined that the maximum β-galactosidase expression was seen ~60 h after transfection.

Immunocytochemistry Screen—The polyclonal antibody raised against bovine brain capillary proteins (10) was depleted of antibodies recognizing common antigens between the BBB and liver and kidney. Rabbit polyclonal antiserum was diluted 1:200 in 0.01 M PBS containing 1 mg/ml bovine serum albumin and adsorbed with rat kidney-acetone powder at 37 °C for 1 h, as described previously (10). After pelleting the kidney-acetone powder, the antiserum was adsorbed with rat liver-acetone powder for 1 h at 37 °C. The subtracted antisera was then diluted further to 1:1000 and adsorbed with fixed COS-1 cell monolayers to remove any additional background binding provided by the COS-1 cells. The subtracted antiserum now only recognized BBB proteins and was ready for the immunocytochemistry screen. COS-1 cell monolayers transfected with the bovine BBB cDNA library were grown for 60 h. The cells were fixed with 5% glacial acetic acid in ethanol. This fixative gave reduced background with the subtracted polyclonal antibody compared with the other fixatives tested including ethanol, methanol, formaldehyde, and paraformaldehyde. The cells were washed with PBS, and endogenous peroxidase activity was quenched with 0.3% H₂O₂ in PBS for 5 min. After washing with PBS again, a blocking solution of 3% goat serum was applied for 30 min. Following three washes with PBS, the endogenous biotin was blocked with an avidin-blocking solution (DAKO, Carpinteria, CA), and washed three times with PBS containing 0.05% Tween 20 (PBST). The kidney- and liver-subtracted BBB-specific polyclonal antibody was used at 1:1000 and incubated with the cells for 1 h at room temperature. The cells were washed three times with PBST and incubated with biotinylated anti-rabbit secondary antibody at 1.5 μg/ml (Vector Laboratories, Burlingame, CA) for 30 min. Subsequent to three more PBST washes, avidin-horseradish peroxidase was added (ABC Elite; Vector Laboratories) for 30 min. The cells were washed three times with PBST, and metal-enhanced diaminobenzidine substrate (Pierce) was added for 5 min. The cell layers were scanned for positive-stained cells using a conventional light microscope. With PBST still covering the cells, a small forceps was used to scrape the immunopositive cells from the dish. This usually required the removal of 10–20 additional neighboring cells for technical purposes. The floating cell layer was then removed using a small pipette tip and added to Hirt extraction buffer for subsequent recovery of the plasmid DNA (see below). To maximize the transfection efficiency of the recovered plasmid DNA into MC1061/P3 cells, no more than 0.02 cm² of cells (~10 small cell sheets) were added to 300 μl of Hirt buffer.

Hirt Extraction and Enrichment of Positive Clones—Plasmid DNA was extracted from the cells with the Hirt procedure (12). Positively stained cells were added to 300 μl of Hirt extraction buffer (100 mM EDTA, 10 mM Tris, pH 8.0, 0.1% SDS, 100 μg/ml proteinase K). After mixing the cells and Hirt buffer, the mixture was incubated overnight at 55 °C. The mixture was centrifuged at 14000 rpm for 2 min to pellet any debris. The plasmid DNA was carefully extracted twice using 1 volume of phenol:chloroform (1:1). Yeast tRNA (10 μg), 1/10 volume of 3 M sodium acetate, and 2.5 volumes of −20 °C ethanol were
added to the extracted aqueous phase to precipitate the DNA and were centrifuged at 14000 × g for 20 min. After washing with 0.5 ml of 70% ethanol, the DNA pellet was dried at room temperature for 10 min and resuspended in 5 μl of 10 mM Tris-Cl, pH 8.5. The entire recovered plasmid DNA sample was then used to electroporate MC1061/P3 bacteria in a single transformation. The enriched pool was subsequently transfected into COS-1 cells and analyzed by immunocytochemistry with the subtracted antiserum, and positive cells were isolated again. This was repeated until a high frequency of positives was in the enriched pool, at which point single clones were selected and analyzed for positive reaction with the subtracted antiserum.

Western Blotting and In Vitro Transcription Translation—COS-1 cells transformed with bovine Lutheran cDNA, pcDNA1.1 control plasmid, and untransfected COS-1 cells were solubilized in non-reducing SDS-polyacrylamide gel electrophoresis buffer (5 mM EDTA, 10% SDS, 400 mM Tris, pH 7.5) and resolved on a 12% SDS-polyacrylamide gel electrophoresis gel followed by transfer of the proteins to a nitrocellulose membrane. The membranes were blocked at 4 °C overnight with TBST (20 mM Tris, 140 mM NaCl, 0.1% Tween 20, pH 7.6) containing 5% dried milk. After washing with PBST, the membranes were incubated with kidney- and liver-subtracted polyclonal or preimmune serum diluted to 1:3000 for 1 h. The membranes were washed, and secondary anti-rabbit horseradish peroxidase conjugate 1:2000 (Sigma) was added for 20 min. The membranes were analyzed with enhanced chemiluminescence substrate (Amersham Bioscience, Inc.) and exposed to x-ray film.

In vitro transcription translation reactions were carried out using the TNT rabbit reticulocyte system under the T7 promoter (Promega). The reactions were carried out with bovine Lutheran plasmid template in both the absence and presence of canine microsomal membranes. The [35S]methionine reaction products were resolved on an SDS-polyacrylamide gel electrophoresis gel, dried, and exposed to film.

DNA Sequencing—DNA sequencing of isolated clones was performed in both directions at the Keck Biotech Resource Laboratory (DNA Sequencing Core Facility, Yale University, New Haven, CT) and at Biotech Core, Inc. (Mountain View, CA). Initial DNA sequencing was performed with standard T7 forward and PCDM8 reverse primers. cDNAs were entirely sequenced in both directions by primer walking with custom synthesized oligodeoxynucleotides, which were purchased from BIOSOURCE International (Camarillo, CA); 17 to 20 mers were designed using the program Oligos 4.0 so that the primers had a Tm > 60 °C in the absence of either stable stem loops or secondary structures. Similarities with other genes in GenBank™ were investigated using the BLAST program (NCBI, National Institutes of Health).

RESULTS

Pure preparations of bovine brain capillaries comprising the BBB were isolated from total brain homogenates and were free of brain cells as shown by light microscopy (Fig. 1A). Messenger RNA was purified from the microvessel preparation using oligo(dT) cellulose, and the integrity of the mRNA was analyzed by Northern blotting with an actin probe. As Fig. 2A demonstrates, full-length mRNA was present, and two mRNA species were observed in the microvessel mRNA preparation. The band at 2.1 kb corresponds to β- and γ-actin, whereas the 1.6-kb band originates from the α-actin component of the smooth muscle cells that line portions of the brain endothelium. Total brain mRNA exhibits only the 2.1-kb band arising from the β- and γ-actin isoforms (Fig. 2A).

The BBB mRNA was then used to create a bovine cDNA library in the pcDNA1.1 vector (see “Experimental Procedures”). This vector promotes expression with the constitutive cytomegalovirus promoter and allows for transient expression without selection pressure. The cDNA library pools consisted of a distribution of supercoiled plasmid sizes (Fig. 2B) that contain cDNA inserts spanning a large range of size (0.6 to 12 kb) upon release via restriction digest (Fig. 2B). The total bovine BBB cDNA library size was 5 × 109, which over-samples the estimated 40,000 protein-encoding genes in the human genome (13) by a factor of 100.
The BBB-specific polyclonal antiserum was raised against bovine endothelial proteins (10). As demonstrated previously, when this antiserum was adsorbed with rat kidney- and liver-acetone powder protein preparations, the depleted antiserum did not specifically recognize any proteins in kidney, liver, or other peripheral tissues (10). Thus, the antibody components that recognize proteins common to brain capillaries, as well as to kidney and liver, were removed. The subtracted antiserum identifies a panel of BBB-specific proteins that have molecular masses ranging from 40 to 200 kDa (Fig. 3, lane 1). This Western blot signal is not observed when the BBB protein is probed with preimmune serum derived from the same rabbit as that which produced the polyclonal antibody (Fig. 3, lane 2). The subtracted antiserum stains brain microvascular endothelial cells and does so in a pattern of continuous staining typical of membrane antigens having endothelial origin (Fig. 1C).

A β-galactosidase expression plasmid under the control of the SV40 promoter was transfected into COS-1 cells to optimize the transfection conditions with DEAE-dextran and determine the proper growth and expression interval for maximum protein expression (see “Experimental Procedures”). The histochemistry with X-gal confirmed transfection of the COS cells (Fig. 1B).

The integrity of the cDNA library was verified by transformation of the library into COS-1 cells, growth and expression for 60 h, and subsequent immunocytochemistry with an anti-α-actin monoclonal antibody (2 μg/ml; American Research Products). As demonstrated in the Northern blot of capillary mRNA, the α-actin transcript is present and thus should be represented in the COS-1 expressed cDNA library. The immunocytochemical labeling of the cDNA library transfected in COS-1 cells demonstrated that the actin cDNA was present and was expressed in the COS-1 cell system (data not shown). In addition, the α-actin clones were likely full-length, because the Kozak sequence required for ribosomal binding and translation initiation needed to be present in the 5′-untranslated region for proper translation of the α-actin transcripts.

Next, 10 positively stained α-actin-transfected COS-1 cells were isolated using a dissecting microscope, and the plasmid DNA was extracted from the cells using the Hirt procedure (see “Experimental Procedures”). This pool was retransfected into COS-1 cells to test for enrichment of the α-actin gene. After this single round of enrichment, the frequency of the actin transcript increased 5-fold. Enrichment was only observed when transfections were performed with the DEAE-dextran method. LipofectAMINE transfections yielded higher transfection efficiencies but did not result in enrichment of the α-actin cDNA, and bacterial pools following extraction and retransfection were 10 times larger. This is presumably because increased cellular uptake of plasmid or nonspecific cell-associated plasmid is recovered in the extraction process and masks the truly positive clones. This did not appear to be a problem when DEAE-dextran was used.

To identify those proteins that elicit the Western blot (Fig. 3A) and intact capillary (Fig. 1C) staining, a modified expression cloning procedure was developed. In this procedure, the differential protein expression derived from the BBB cDNA library was evaluated using the BBB-specific subtracted antiserum (Fig. 1). The library was transfected into COS-1 cells and subjected to immunocytochemistry 60 h later. The immunocytochemistry process was optimized for background reduction (see “Experimental Procedures”), because the recog...
nition of multiple antigens by the antiserum increases the background significantly over that seen with monoclonal antibodies or polyclonal antibodies raised against a single antigen. A typical first round immunopositive cell is shown in Fig. 4A, and 13 potential positive cells were extracted from the plate, and the plasmid DNA was recovered and retransfected into COS-1 cells. After repeating the immunocytochemistry with the BBB-specific antiserum, a 33-fold enrichment was observed, and a representative field is shown in Fig. 4B. At this point, positive clones were again isolated, and the process was repeated to yield a highly enriched pool of 150 clones (see Fig. 4C and Fig. 1D). Plasmid DNA from 36 of these clones was transfected into COS-1 cells, and three clones that resulted in positive staining with the BBB-specific polyclonal antibody were identified (Fig. 4D). These clones did not react with the preimmune antiserum (Fig. 4E) and represented clones that were reactive only with the BBB-specific antibody mixture. Only three rounds of enrichment were necessary to clone this protein whose cDNA is estimated to be present at a frequency of 1 in 5000 in the bovine cDNA library.

The clones were sequenced and found to encode bovine Lutheran membrane glycoprotein. The 2.4-kb cDNA insert was full-length and consisted of a 39-nucleotide 5'-untranslated region, a 1887-nucleotide open reading frame including a stop codon, and a 434-nucleotide 3'-untranslated region with a 38-nucleotide poly(A) tail (Fig. 5). The putative methionine translational start codon yielded a protein of 641 amino acids having 77% amino acid identity with that of human Lutheran glycoprotein and 69% amino acid homology to both rat and mouse Lutheran glycoprotein. Based on homology to the human protein, a mature transmembrane protein of 597 amino acids results (Fig. 5).

The cloned plasmid encoding the bovine Lutheran gene was transfected into COS-1 cells, and the lysates of cell protein were analyzed by non-reducing gel electrophoresis and Western blotting with the BBB-specific polyclonal antiserum (Fig. 6A). The Lutheran protein encoded by the plasmid had a molecular size of 80 kDa, and the protein could not be detected in lysates of control plasmid-transfected cells or untransfected cells (Fig. 6A). Labeling with preimmune serum was also negative (data not shown). In vitro transcription/translation reactions with the bovine Lutheran plasmid as a template yielded a core protein size of 67 kDa whereas reactions including the post-translation processing machinery of canine microsomal membranes resulted in glycosylation and increased size of 71–77 kDa (Fig. 6B).

**DISCUSSION**

The subtractive expression cloning methodology described in this study was used to identify the Lutheran glycoprotein as a BBB-specific protein not found in liver or kidney. The Lutheran glycoprotein is a glycosylated membrane protein that can have reduced solubility in 2-dimensional gel electrophoresis of cell extracts (5), and cloning of this gene confirms the ability of the methodology to clone genes encoding membrane proteins. The Lutheran glycoprotein is one of many

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**Fig. 4. Immunocytochemical expression cloning and enrichment using liver-/kidney-subtracted BBB-specific antiserum.** A, original cDNA library transfected into COS-1 cells showing a single positive cell. B, first round enriched pool showing four immunopositive cells. C, second round enriched pool showing many immunopositive cells. D, final enriched pool, where all transfected cells are immunopositive. E, final enriched pool immunostained with the preimmune serum. Panels A–D are labeled with subtracted BBB-specific antibodies, whereas panel E is labeled with preimmune serum. Bar represents 200 microns.
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proteins expressed in the brain microvessel that react with the kidney-/liver-absorbed polyclonal antiserum (Fig. 3, lane 1). The genes encoding additional brain vascular-specific proteins may be cloned with further rounds of immunoscreening using the subtractive antibody expression cloning methodology.

The bovine Lutheran glycoprotein is a membrane protein that is involved in basal cell adhesion (14) and contains several immunoglobulin-like domains with the membrane proximal domain functioning as a laminin receptor (15). Lutheran protein is very similar to the B-CAM adhesion protein found on the basal surface of epithelial cells having only an additional 40-amino acid cytoplasmic tail at the carboxyl terminus of the protein (16, 17). The protein cloned in this study was definitively the Lutheran glycoprotein and not B-CAM, as it extends beyond Pro557 (Fig. 5), which is the carboxyl terminus of the B-CAM protein (16). Moreover, the cDNA was sized at 2.4 kb rather than the 4.0-kb characteristic of human B-CAM (18). Lutheran is a glycosylated protein, and the bovine sequence has three consensus N-linked glycosylation sites and leads to a COS-1-produced protein of 80 kDa. The in vitro transcription translation reactions indicated a core protein size of 67 kDa, similar to the predicted molecular mass of 65 kDa for bovine Lutheran. The conservation of 10 extracellular cysteine residues that contribute to the immunoglobulin folds of the protein was also observed (Fig. 5). The Lutheran protein has been demonstrated to be BBB-enriched, and Northern blotting has illustrated that the transcript is not detectable in either the liver or kidney and can be used as a marker of endothelial cells of the blood-brain barrier (19).

This methodology proved effective in cloning the Lutheran glycoprotein, although it is a glycosylated transmembrane protein. Membrane proteins are inherently more difficult to produce as recombinant proteins than secreted proteins, and the eukaryotic post-translational processing machinery and quality-control apparatus is an advantageous asset of this methodology in comparison to similar prokaryotic systems. The method is particularly attractive for cloning tissue-specific membrane proteins and can be easily applied to other cell and tissue types. In addition to comparing different tissue types, this subtractive process can be adapted to analyzing the differential protein expression for tissues under different physiological conditions or in different growth states. This technology has the potential to be the basis for tissue-specific proteomics. In particular, this method can be used for the study of organ-specific vascular proteomics, and the present study describes the application of the methodology to the study of brain vascular proteomics. In parallel, brain vascular functional genomics can be investigated following the initial isolation of brain capillary-derived mRNA and the application of subtractive hybridization techniques, as recently described (21). The information obtained with both vascular genomics and vascular proteomics platforms is complementary and provides a complete analysis of tissue-specific gene expression at the microvasculature.

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**The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™EBI Data Bank with accession number(s) AF270512.**

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**Fig. 5. Sequence for full-length cDNA encoding bovine Lutheran glycoprotein.** Putative leader peptide is *underlined*. Conserved cysteine residues are in *bold italics*. Consensus N-linked glycosylation sites are denoted in *bold* and *underlined*. The transmembrane domain is *underlined* and in *italics*. Pro557 represents the carboxyl terminus of the B-CAM protein. Amino acid numbering is based on homology with the human Lutheran glycoprotein and is based on Glu1 as the amino terminus of the mature protein (20). The 5' portion to Ser242 was determined in this study and combined with the 3' portion extending from Gin543 to the poly(A) tail that was described previously (19).

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**Fig. 6. Size determination of the bovine Lutheran protein.** A, non-reducing Western blot of COS-1-transfected cell extracts. Lane 1, bovine Lutheran plasmid. Lane 2, pcDNA1.1-negative control. Lane 3, untransfected COS-1 cells. The membrane was probed with the liver-/kidney-subtracted BBB-specific antiserum. B, [35S]methionine-labeled in vitro transcription translation products. Lane 1, bovine Lutheran plasmid template. Lane 2, bovine Lutheran plasmid template in the presence of canine microsomal membranes. Lane 3, pcDNA1.1 plasmid template in the presence of canine microsomal membranes.
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