Subtractive Expression Cloning Reveals High Expression of CD46 at the Blood-Brain Barrier

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Abstract. A subtractive expression cloning methodology was used to identify proteins having enriched expression at the blood-brain barrier (BBB) in comparison to liver and kidney tissues. A bovine brain capillary COS-1 cell cDNA expression library was screened with a BBB-specific antiserum. This strategy revealed that the membrane cofactor protein CD46, which is a regulator of complement activation in vivo and is also a potential measles virus receptor, is highly expressed at the BBB. The selective CD46 expression in brain at the BBB was confirmed by Northern blot analysis and confocal microscopy. The finding of selective expression of CD46 at the BBB is consistent with an important role played by the microvasculature in the immune surveillance of the brain.

Key Words: Blood-brain barrier; CD46; Measles.

INTRODUCTION

The microvasculature plays an important role in the immune surveillance within the central nervous system (CNS) (1). Endothelial cells play a role in lymphocyte homing to the brain (2), and microvascular pericytes express class II histocompatibility antigens (3, 4). The class II histocompatibility antigen has been recently cloned from a rat brain microvascular cDNA library (5). The expression of other components of the immune system at the brain microvasculature could be elucidated by the use of a subtractive expression cloning methodology recently described (6). This methodology was used in the present study in conjunction with an antiserum raised against freshly isolated brain microvessels (7) to identify proteins having enriched expression at the blood-brain barrier (BBB) compared to peripheral tissues such as kidney or liver. This antiserum is adsorbed with liver and kidney proteins to remove the antibodies that recognize proteins with similar expression profiles between the BBB and peripheral organs. The antibodies that remain are then used in an expression cloning procedure to extract genes from a BBB cDNA library that encode for proteins having enriched expression at the BBB. This method identified the CD46 membrane cofactor protein, a regulator of complement activation in vivo, as a BBB-enriched protein. The cloned gene product encoding bovine CD46 is an isoform typically found in brain having just 1 serine/threonine/proline (STP) domain, and has a distinct cytoplasmic tail previously found in human sperm and placenta (8). The elevated expression of the CD46 gene at the BBB was confirmed by Northern blotting and confocal microscopy using a CD46 antibody.

MATERIALS AND METHODS

Expression Cloning

Bovine brain capillary mRNA was isolated as previously described (9). This mRNA was used to create a cDNA library (Superscript II, Invitrogen, San Diego, CA) with BstXI and NotI flanking sites for directional cloning into the pcDNA1.1 expression plasmid (Invitrogen). The cDNA library was transfected into confluent monolayers of COS-1 cells (American Type Culture Collection, Rockville, MD) using the diethylaminoethyl (DEAE) dextran method, and transient gene expression controlled by the cytomegalovirus (CMV) promoter was carried out for 60 hours (h) (6). Immunocytochemistry was performed as described below and positively stained cells were lifted from the plates using a dissecting microscope. Plasmid DNA was recovered from these isolates of positive cells using Hirt extraction (10) and the enriched pools were re-transfected into COS-1 cells. One of the positive pools consisted of about 400 clones. This pool was manually subdivided into 8 new pools. Single clones from the pool with the highest level of positive signal were then analyzed for positive staining. Seven positive clones were identified and sequenced revealing identical replicates of the bovine CD46 gene.

Immunocytochemistry

A polyclonal antiserum raised against bovine endothelial protein (7) was adsorbed with rat liver and kidney powders to subtract antibodies to common antigens. The acetone powder was suspended in the antiserum, which was diluted 1:200 in 0.01 M phosphate buffered saline (PBS) plus 1 mg/mL bovine serum albumin (BSA) and incubated for 1 h at 37°C. The subtracted antiserum was then diluted to a working concentration of 1:1,000 and adsorbed with a monolayer of COS-1 cells to remove background antibody binding. Cells transfected with the bovine cDNA library were fixed with 5% acetic acid in ethanol for 10 min. After washing with PBS, endogenous horseradish peroxidase activity was quenched with 0.3% hydrogen peroxide in PBS for 5 min. The fixed cells were then blocked with 3% goat serum and endogenous biotin was blocked with biotin block solution (DAKO, Carpinteria, CA). The subtracted antiserum was then applied for 1 h at room temperature. After PBST (PBS plus 0.05% Tween-20) washing, a biotinylated antirabbit secondary antibody was applied for 30 min (1.5 mg/L in PBS plus BSA, Vector Labs, Burlingame, CA). The cells were...
washed with PBST and ABC Elite avidin/horseradish peroxidase conjugate was added to the cells for 30 min. Subsequent to another set of PBST washes, metal enhanced diaminobenzidine (DAB) substrate (Pierce Chemical Co., Rockford, IL) was added and the development reaction allowed to progress for 5 min. Immunostained cells were identified by scanning culture dishes with light microscopy.

**Western Blotting**

COS-1 cells transfected with the bovine CD46 plasmid or pcDNA1.1 control or untransfected COS-1 cells were solubilized with non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (5 mM EDTA, 10% SDS, 400 mM Tris, pH 7.5) to a concentration of 3 μg/μL total protein. Protein extracts from intact bovine capillaries were also prepared in non-reducing SDS-PAGE buffer. The samples were resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose. The blots were blocked overnight at 4°C with 5% dry non-fat milk in TBST (20 mM Tris, 140 mM NaCl, 0.1% Tween-20 pH 7.6). Primary antibody was applied for 1 h at room temperature and consisted of kidney and liver adsorbed polyclonal antiserum diluted to 1:3,000 in TBST or preimmune antiserum, which was also adsorbed and diluted to 1:3,000. After TBST washing, an anti-rabbit horseradish peroxidase conjugate (Sigma Chemical Co.) was applied for 20 min diluted to 1:2,000. The blots were then developed with enhanced chemiluminescence (ECL) substrate (Amersham, Chicago, IL) and exposed to x-ray film.

**In Vitro Transcription/Translation**

In vitro transcription/translation reactions were performed using a rabbit reticulocyte system under the T7 promoter (Promega, Madison, WI). A titration of starting CD46 plasmid concentration (0.125–0.5 μg) was used along with 32P-labeled methionine as a probe. A reaction having pcDNA1.1 without insert was also used as a negative control. After a 90-min incubation of the reticulocyte reaction mixture, 2 μL of the 25 μL reaction was resolved by 12% SDS-PAGE. The gel was fixed (50% methanol, 10% glacial acetic acid in water) for 30 min and incubated for 5 min prior to gel drying (7% methanol, 7% acetic acid, and 1% glycerol in water). The gel was dried for 45 min at 80°C and exposed to x-ray film.

**Northern Blotting**

One microgram each of bovine brain capillary mRNA and total bovine brain mRNA was resolved on a 1.5% agarose/formaldehyde gel. The mRNA was transferred to a Genescreen plus membrane (Perkin Elmer, Boston, MA) using the paper towel method (11). After baking to bind the mRNA to the membrane, the membrane was prehybridized at 42°C for 2 h (50% formamide, 2× Denhardt’s, 2× SSPE, 1% SDS, 0.2 mg/mL salmon sperm DNA). The probe was made with the entire CD46 cDNA by using HindIII and XhoI restriction digest of the bovine CD46 plasmid clone. Then, using the method of random priming (Megaprime Kit, Amersham), 32P-dCTP labeled CD46 probe was produced and 6 × 106 cpm of probe was added to the prehybridization solution containing 5% dextran sulfate. After binding to the filter overnight at 42°C, the membrane was washed stringently and exposed to film at ~80°C. Similarly, after stripping the membrane, the membrane was re-probed with an actin probe that identifies both the alpha and beta actin isoforms.

**DNA Sequencing**

DNA sequencing of isolated clones was performed in both directions at the Keck Biotech Resource Lab (DNA Sequencing Core Facility, Yale University, 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, NIH).

**Confocal Microscopy**

Frozen sections (20 μm) of brains from adult rats were fixed in 100% acetone at −20°C for 20 min. After washing in PBS, sections were blocked with 10% horse serum for 30 min. Sections were then incubated with (a) 10 μg/mL rabbit anti-human/rodent CD46 (Research Diagnostics, Flanders, NJ) and 10 μg/mL goat anti-GFAP (GFAP = glial fibrillary acidic protein) (Research Diagnostics), or (b) 10 μg/mL rabbit anti-human/rodents CD46 and 2 μg/mL goat anti-Glut1 glucose transporter (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing in PBS, sections were incubated with Rhodamine-conjugated donkey anti-goat IgG 1:200 (Research Diagnostics) for 30 min. After another wash with PBS, sections were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG 1:100 (Sigma) for 30 min. Sections were then washed in PBS and the coverslips were mounted on slides with 5% n-propyl gallate in 100% glycerol. The sections were examined on a Zeiss LSM 5 Pascal confocal laser scanning microscope attached to a Zeiss axioskop 2 microscope using epifluorescence. Serial plane images were collected at 0.32- or 0.64-μm intervals at a given depth of the fluorescent preparation, and images were reconstructed using the Zeiss LSM software.

**RESULTS**

The polyclonal antiserum raised against bovine BBB membrane proteins was adsorbed with kidney and liver acetone protein powders to remove antigenicity to BBB proteins that are also expressed in the liver and kidney. The depleted or subtracted antiserum was then used to screen a bovine cDNA library transfected into COS-1 cells. Immunocytochemistry revealed positively stained cells (Fig. 1A) at a low frequency (1 in 300,000 cells). The positively stained cells were isolated under a dissecting microscope and the plasmid DNA recovered with the Hirt procedure (Materials and Methods). Subsequently, this pool enriched for BBB-specific proteins was re-transfected into COS-1 cells and assayed again. Enriched positive signal was confirmed and manual subpooling was
performed with the enriched pool for 2 additional rounds until single positive clones were isolated (Fig. 1B). The staining pattern indicated diffuse cytoplasmic staining in addition to membrane labeling with some punctate staining visible at higher magnification (Fig. 1C).

The 7 positive clones were sequenced and each clone was found to code for bovine CD46. The full-length 1,344 nucleotide cDNA consisted of a 35 nucleotide 5’ untranslated region, a 1,098 nucleotide open reading frame, and a 211 nucleotide 3’ untranslated region including a 25 nucleotide polyA tail (Fig. 2). The putative start codon reveals a 42 amino acid signal peptide and a 365 amino acid mature protein. The protein contains the 4 characteristic short consensus repeats (SCR1–4) that are involved in binding complement (12, 13) and measles virus binding (12, 14, 15). The repeats are followed by a single serine/threonine/proline rich region that is alternatively spliced and can consist of up to 3 of these domains (STP A, B, C) (8, 16–18), a transmembrane domain, and a short cytoplasmic tail (Fig. 2). The 3 N-linked glycosylation sites found in human CD46 are conserved in bovine CD46 and several potential O-linked glycosylation sites are present in the STP-rich region. The amino acid similarity from the signal peptide up to the putative transmembrane domain between bovine CD46 and porcine, monkey, or human CD46 is 57%, 49%, and 48%, respectively.

The size of the protein encoded by the bovine CD46 gene was confirmed by Western blotting using the subtracted antiserum and COS-1 cell transfectants. As Lane 2 of Figure 3A demonstrates, the size of the bovine CD46 protein is heterogeneous and centered around 50 kDa.
Fig. 2. Full-length sequence of bovine CD46. The deduced amino acid sequence is indicated starting with the methionine initiation codon. The putative signal peptide is indicated in boldface. The short consensus regions (SCR) are depicted above the sequence and are followed by the serine/threonine/proline region in boldface italics. The transmembrane region is underlined. The cytoplasmic tail is denoted CYT3 for cytoplasmic tail 3. Conserved potential N-linked glycosylation sites are identified with a ↑.

J Neuropathol Exp Neurol, Vol 61, July, 2002
Fig. 3. Determination of protein size for bovine CD46 with non-reducing SDS-PAGE analysis. A: Lane 1: 20 μg of bovine brain capillary protein; lane 2: 30 μg of bovine CD46 transfected COS-1 cell protein; lane 3: 30 μg of pcDNA1.1 transfected COS-1 cell protein as negative control; lane 4: 30 μg of untransfected COS-1 cell protein as negative control. The membrane was probed with the subtracted BBB-specific polyclonal antiserum that had been pre-absorbed with acetone powders of rat kidney and liver. An identical blot was probed with preimmune serum and no specific bands were detected in any of the lanes (data not shown). B: Autoradiograph of 35S-methionine labeled in vitro transcription/translation products. Lane 1: template is bovine CD46 plasmid; lane 2: template is pcDNA1.1 plasmid negative control.

Fig. 4. Northern blot analysis of BBB enrichment of CD46 transcripts. A: Membrane probed with CD46 probe. B: Same membrane is probed with a radiolabeled actin cDNA. Lane 1: 1 μg total bovine brain mRNA; lane 2: 1 μg bovine brain capillary-derived mRNA. Filters were exposed to Biomax MS film (Kodak, Rochester, NY) for 8 and 24 h at −80°C for CD46 and actin, respectively.

Confocal microscopy indicated that in addition to enriched CD46 mRNA expression, the actual protein expression of CD46 was also enriched at the brain endothelium (Fig. 5). Co-localization experiments were performed to define the endothelial origin of the CD46 protein. In the first co-localization experiment, labeling of CD46 was compared with that of Glut1, a highly localized brain endothelial-specific glucose transporter. The CD46 was observed to co-localize with this endothelial marker as well as being observed in astrocyte processes (Fig. 5A–C). The second co-labeling experiment was performed with antibodies to GFAP, a marker of astrocyte processes. This showed that the CD46 co-localized with GFAP but also could be seen outlining vessels, distinct from the signal derived from astrocyte staining (Fig. 5D–F). A brain capillary preparation invariably includes astrocyte foot processes as these intimately invest the cerebral endothelium. This study illustrates that although CD46 is seen in perivascular astrocyte foot processes, the endothelium is also enriched in CD46 expression.

DISCUSSION

The subtractive expression cloning methodology used in this study was effective in cloning a protein, CD46, which is enriched at the BBB, relative to whole brain. This method is especially useful because it does not require any a priori knowledge of a differentially expressed protein target, but yet can identify those proteins that are enriched in comparison to other tissues, in this case kidney and liver. Also, the subtractive expression cloning method utilizes the direct readout of increased protein expression, not simply the presence of increased levels of mRNA (6). The CD46 isoform cloned in this study is

The heterogeneity likely arises from differential glycosylation of the CD46 protein. The subtracted antiserum recognizes a panel of BBB-specific proteins ranging in size from 40 to 200 kDa (lane 1, Fig. 3A), and one of these proteins corresponds to the CD46 protein, as determined by the alignment of the BBB bands and the CD46 protein produced in COS-1 cells (lanes 1 and 2, Fig. 3A). An in vitro transcription/translation reaction in the absence of post-translational machinery also confirmed the size of the unglycosylated core protein subunit as being 50 kDa (Fig. 3B).

Northern blotting of total bovine brain and bovine brain capillary-derived mRNA with the full-length CD46 clone indicated that the CD46 mRNA was abundantly expressed at the BBB in comparison to the surrounding brain tissue (Fig. 4A). The level of the CD46 mRNA at the BBB was high, as the CD46 mRNA was detected with only an 8-h exposure of the x-ray film (Fig. 4). Although the 1.35-kb transcript was cloned from the cDNA library, the major CD46 transcript at the BBB is 4.2 kb in size and the smaller 1.35-kb transcript was not detected. The observed difference in CD46 mRNA levels is not a result of unequal mRNA loading, since the levels of beta actin mRNA (2.1 kb) are nearly equivalent (Fig. 4B). The typical pattern of a BBB mRNA preparation is apparent with a second band at 1.6 kb that corresponds to the alpha actin component derived from smooth muscle cells present in the microvessel preparation.
the β-isoform containing only 1 of 3 possible STP domains that are extensively O-glycosylated. This causes the molecular size of the protein to be smaller (50–56 kDa) than the α-isofrom of CD46 that contains 2 STP domains and has a distinct molecular size of (58–68 kDa) (8, 16, 17). The β-isofrom cloned in this study is the predominant brain isofrom and is a result of differential splicing of exon 8 in humans (19). The bovine CD46 gene appears to have a similar pattern of splicing, or at least a similar tissue distribution of isofoms as that observed with the human CD46 gene.

Although CD46 expression is widespread in pigs, monkeys, and humans, there is limited CD46 expression in rodents, and no CD46 mRNA is detectable in rat kidney and liver on Northern blotting (20, 21). RT-PCR is necessary to detect the CD46 transcript in these tissues (20). The limited distribution of CD46 in liver and kidney explains why anti-CD46 antibodies were not removed when the anti-bovine BB antiserum was absorbed with acetone powder of rat liver and kidney (Materials and Methods).

The major transcript seen at the BBB is 4.2 kb in size although a 1.35-kb transcript was cloned from the BBB cDNA library. The CD46 transcripts observed by Northern blotting in the rat and mouse are 1.3–1.6 kb (20–22), and the mouse also exhibits a less intense 4.0-kb band in the testis (22). Similar to the present study, the human CD46 gene was cloned as a 1.5-kb transcript; however, Northern blotting indicated a 4.2-kb band for CD46 transcripts in the human U937 and HeLa cell lines (23). The difference in size between the 4.2- and 1.35-kb transcripts is a result of alternative processing of the mRNA, which leads to different 3′-untranslated regions (UTR) in the transcripts. This may be a result either by the use of an alternative, earlier polyadenylation site or by alternative splicing of the RNA product. Only 1 polyadenylation signal is present in the region 10–30 bases upstream of the polyA tract and this AAAAAA site has been shown to have <5% of the processing efficiency of the canonical AATAAA signal (24). It is possible that the predominant 4.2-kb transcript may make use of a more efficient signal downstream from the polyadenylation site of the 1.35-kb transcript. Alternative splicing is also suggested by the observation that the homology of the transmembrane region and cytoplasmic tails was significantly reduced compared to the SCR regions. Most commonly, human CD46 contains 1 of 2 cytoplasmic tails (CYT1, CYT2) created by alternative splicing of exon 13, whereas the brain preferentially expresses CYT2 (17, 25). However, certain deletions of exon 12 give rise to a third cytoplasmic tail (CYT3) that is homologous to that seen in bovine CD46 (64% amino acid identity). This cytoplasmic tail has been observed in human sperm and placental cDNA clones (8). Even though the bovine and human genes may differ significantly in terms of genomic
sequence, the presence of CYT3 in the bovine CD46 clone points to a possible alternative splicing event.

The increased expression of CD46 at the BBB may indicate a role in the pathogenesis of complications stemming from CNS invasion by the measles virus. It is possible that the measles virus gains access to the CNS by binding the CD46 receptor and undergoing transport across the brain capillary endothelium. In human patients with acute fatal measles, infection of cerebral endothelial cells with measles virus was observed while no neuronal or glial infection could be detected (26). In contrast, chronic measles virus encephalitis is associated mainly with neuronal and glial infection by the virus (26–30). In addition, the CD46 protein levels are greatly reduced in the endothelial cells, neurons, and astrocytes of encephalitic brains where lesions occur, while CD46 levels in unaffected parts of the brains are normal (28). These results suggest that BBB expression of CD46 may be involved in the etiology of measles virus complications of the CNS.

An alternative measles virus receptor is the signaling lymphocyte-activation molecule (SLAM), also known as CDw150, and SLAM is primarily found in lymphocytes and dendritic cells with barely detectable levels in the brain (31, 32). Although the SLAM protein has been implicated as the receptor required for primary infection of lymphocytes with non-Edmonston strains of the measles virus (33), CD46 may still play a role in the secondary expansion of the virus into other tissues, including the CNS. It is possible for single amino acid changes in the hemagglutinin protein of the measles virus to alter receptor recognition from SLAM to CD46 (34, 35). Such mutations that change the hemagglutinin binding specificity can occur during viral replication and could lead to further widespread viral transfer to other tissues. This secondary infection could proceed via the CD46 protein, although primary infection may require interaction with the SLAM protein (33).

Transgenic mice harboring the gene for human CD46 as a full genomic sequence insertion, or as a single CD46 isoform under a neuronal specific promoter, are susceptible to the measles virus (36–38) and subsequent neurological disease and death upon intracerebral injection of the virus. The CD46 and not the antiviral immune response is the cause of the neurological disease in mice (39). As the disease state of the measles virus progresses in the CD46 transgenic mice, the infiltration of T and B-lymphocytes and macrophages into the brain has been observed. Coordinate increases in cytokine signals and MHC class I and II molecules occurs, with the upregulation of MHC molecules occurring primarily on the endothelial cells (40). These combined findings suggest an important role for the BBB CD46 in the pathogenesis of measles infection in the brain.

The finding of selective expression in brain of CD46 at the microvasculature is further evidence that important interactions take place between the brain and the immune system at the BBB. Components of the immune system that are selectively expressed in brain at the microvasculature include the MHC class I (5) and II (3) receptors, the neonatal immunoglobulin Fc receptor, FcRn (41), and the CD46 membrane cofactor protein, which is a regulator of complement activation in vivo. The selective expression in the brain of these immune effector molecules at the BBB suggests the microvasculature may be a primary site of initial immune activation within the CNS.

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Received November 26, 2001
Revision received March 19, 2002
Accepted March 19, 2002.