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BSMAP, a Novel Protein Expressed Specifically in the Brain Whose Gene Is Localized on Chromosome 19p12

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Using the sequence of cosmids derived from chromosome 19p12, we have identified a gene encoding a novel protein, BSMAP (brain-specific membrane-anchored protein) and cloned cDNA encoding the full-length open reading frame. Northern blot analysis revealed that BSMAP mRNA is preferentially expressed at a high level in the brain. BSMAP has a putative transmembrane domain and is predicted to be a type-I membrane glycoprotein. Genomic sequence analysis revealed that the gene encoding BSMAP consists of eight exons spanning approximately 8 kb and lies 6 kb away from the gene encoding CLF-1 in a reverse orientation. Although no candidate genetic disorders were found to map either to this precise region of chromosome 19 or to the syntenic region of the mouse genome, the highly specific expression of BSMAP mRNA suggests a role for the protein in CNS function.

Although being short, (approximately 60 megabases) human chromosome 19 has a relatively high GC content (1) and gene density compared to other chromosomes and also has an abundance of Alu repeats (2). It has been well characterized at the genetic level, with a number of genes giving rise to the pathogenesis of various human diseases being mapped on this chromosome. These include amongst others the low density lipoprotein receptor (LDL-R) implicated in familial hypercholesterolemia (3), the erythropoietin receptor (EPOR) implicated in primary familial and congenital polycythemias [PFCP (4)] and apolipoprotein E (APOE) playing a role in late onset familial Alzheimer's disease (5).

The Human Genome Center at the Lawrence Livermore National Laboratory, California, has undertaken a project to construct a "high resolution, sequence ready" integrated metric physical map of human chromosome 19. Construction of this map primarily involves the sequencing and fingerprinting of chromosome 19-specific bacterial-based cosmid, BAC, PAC, and P1 clones, which are overlapped to generate contigs. The contigs are subsequently ordered (6).

The gene for the type I cytokine receptor homologue CLF-1 (7) was recently localized to chromosome 19 using the sequence derived from a chromosome 19p12-specific cosmid R30292, which was sequenced at the Human Genome Center. Using the CLF-1 cDNA sequence to query the GenBank database, all nine CLF-1-specific exons were identified within the cosmid sequence. In addition to these exons, we observed that seven other putative exons had been identified by bioinformatics, and were situated adjacent to those of CLF-1 but coding in the reverse orientation. Using sequence information derived from these putative exons within cosmid R30292 and that of the overlapping cosmid R30085 (also sequenced at the Human Genome Center), we have identified a new gene which we have named brain-specific membrane anchored protein (BSMAP) and which has been given the approved symbol *C19orf4* by the human gene nomenclature committee. The cloned cDNA encodes a putative 40-kDa membrane-bound type-I glycoprotein whose mRNA is strongly and preferentially expressed in the CNS. BSMAP shares significant homology with the amino acid sequence derived from a novel cDNA cloned from $CD34^+$ hematopoietic progenitors, although their respective mRNA tissue distribution appears to be somewhat different. We failed to identify any genetic disease implicating CNS function which have been mapped to this precise region of chromosome 19. One may speculate however that such a highly preferential tissue expression of BSMAP mRNA suggests a role for this protein in brain function.

The BSMAP cDNA sequence has been deposited in the GenBank database under Accession No. AF186264.

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MATERIALS AND METHODS

Source of cells and culture conditions. The fibroblastic cells lines HEK 293 and COS1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's modified Eagle's medium and Ham's F12 nutrient mix supplemented with 10% FCS.

Sequencing and sequence analysis. The sequence of the cosmid R30292 is deposited in the GenBank database with Accession No. AC003112. The sequence of the cosmid R30085 was obtained from the Lawrence Livermore national laboratory (LLNL) human genome center site on the Internet (http://bbrp.llnl.gov). Exon predictions were performed using GRAIL 2 (8) and through sequence similarity with ESTs. cDNA sequencing reactions were performed using the ABI Prism BigDye Terminator DNA sequencing kit (Perkin–Elmer Applied Biosystems, Warrington, UK) following manufacturer's guidelines. Nucleotide and predicted amino acid sequence homologies were analyzed using the CLUSTAL W program (9). Signal peptide predictions were performed using the SignalP program (http://www.cbs.dtu.dk/services/SignalP) and transmembrane region predictions performed using both the TMpred (http://www.isrec.isb-sib.ch/) and TMHMM (http://genome.cbs.dtu.dk) programs respectively.

cDNA cloning. HEK 293 first-strand cDNA was synthesized using 2 μg of poly(A)$^+$ RNA by reverse transcription using an oligo-dT primer (Pharmacia, Uppsala, Sweden). BSMAP cDNA containing the full length ORF was amplified from cDNA corresponding to 10 ng of poly(A)$^+$ RNA by PCR using the Advantage-GC cDNA PCR kit (Clontech Laboratories, Inc., Palo Alto, CA) with the primers 5′-ATGGCTGCGGTGGCGCTGAT-3′ and 5′-GGCCTACAGCTTGGTCAGGT-3′ following the manufacturer's guidelines. Amplified cDNA products of the appropriate size (1031 bp) were directly cloned into the TA cloning vector pGEM-Teasy (Promega Corp., Madison, WI).

Northern blot analysis. The 1031-bp BSMAP cDNA fragment cloned in the plasmid pGEM-Teasy was excised by digestion with the restriction enzyme *Eco*RI and purified. The fragment was subsequently radiolabeled with [α-^{32}P]dCTP (220 TBq/mmol; Amersham France SA, Les Ulis, France) using the random priming method (10) and used to probe the human 12-lane multiple tissue Northern and human RNA Master blots (Clontech). Hybridizations were performed in ExpressHyb solution (Clontech). Hybridization and washing conditions were carried out according to the manufacturer's guidelines.

Transient transfection. The BSMAP cDNA cloned in pGEM-Teasy was reamplified by PCR using the Advantage-GC cDNA PCR kit with the primers 5′-CCCAAGCTTACCATGGCTGCGGTGGCGCTG-3′ and 5′-CCGCTCGAGGGCCTACAGCTTGGTCAGGT-3′. The amplified cDNA product was cloned into the mammalian expression vector pCDNA3 (Invitrogen, Leek, The Netherlands) using the restriction enzymes *Hin*dIII and *Xho*I. To generate a modified form of BSMAP containing a C-myc antibody recognition tag (11) within the predicted extracellular region of the protein, a portion of the BSMAP cDNA cloned in pGEM-Teasy was amplified by PCR using the Advantage-GC cDNA PCR kit with the primers 5′-GAATTCACTAGTGATTTATGG-3′ and CGCGGATCCCCAGATCCTCTTCTGAGATGAGTTTTTGTTCGATCA-GAACGGCTCTTCAT-3′. The resulting PCR fragment was recloned into the pGEM-Teasy/BSMAP cDNA construct using the restriction enzymes *Spe*I and *Bam*HI. This modified BSMAP cDNA was subsequently reamplified by PCR using the Advantage-GC cDNA PCR kit with the primers 5′-CCCAAGCTTACCATGGCTGCGGTGGCGCTG-3′ and 5′-CCGCTCGAGGGCCTACAGCTTGGTCAGGT-3′ and cloned into the mammalian expression vector pCDNA3 using the restriction enzymes *Hin*dIII and *Xho*I. The CD24 coding sequence (omitting the signal peptide coding region) was amplified by PCR from PBMC derived cDNA using the primers 5′-AATTGAATTCAACGCAGATTTAT-TCCAGTG-3′ and 5′-AATTGATATCTTAAGAGTAGAGATGCAGAA-3′. The amplified cDNA was cloned into the expression vector pSecTag2C (Invitrogen) in frame with the upstream Igκ signal peptide and C-myc antibody recognition tag using *Eco*RI and *Eco*RV. COS1 cells were transfected with BSMAP and CD24 expression constructs using the liposome transfection reagent FuGENE 6 (Boehringer-Mannheim) following manufacturer's guidelines. In all cases, 1.5 μg of DNA and 6 μl of FuGENE 6 reagent were used per transfection.

Western blot analysis. At 48 h posttransfection, COS1 cells and their supernatants were harvested. Cells were washed twice with 1× PBS, resuspended in lysis buffer (25 mM Hepes, 1% Triton-X-100, 1 mM EGTA, 100 mM NaCl, 10% glycerol) at a concentration of 1×10^7 cells/ml and insoluble material subsequently removed by microcentrifugation. Cell supernatants were centrifuged at high speed [35,000 rpm with a SW60Ti rotor (Beckman Instruments Inc., Fullerton, CA)] for 30 min to remove cellular debris. Cell lysates and supernatants were diluted 1:1 in tris-glycine-SDS sample buffer containing 750 mM 2-Mercaptoethanol, heated to 95°C for 5 min, migrated on a 10% SDS–polyacrylamide gel and electrotransferred to a nitrocellulose membrane (12). Membranes were blocked in 1× PBS containing 5% dried milk and 0.1% Tween 20 for 1 h at room temperature followed by a subsequent 1 hour incubation in the same buffer containing the anti-C-myc tag monoclonal antibody at 5 μg/ml. Bound antibody was detected using horseradish-peroxidase labeled sheep anti-mouse Ab and ECL (Amersham France, SA) following the manufacturer's guidelines.

Flow cytometric analysis. COS1 cells were harvested 48 h post-transfection, washed once in staining buffer (1× PBS, 1% BSA, 0.01% sodium azide) and successively incubated for 30 min with the anti-C-myc tag monoclonal antibody at 25 μg/ml in staining buffer and FITC-labeled sheep anti-mouse F(ab′)$_2$ fragments (Silenus Laboratories, Hawthorn, Australia) diluted 1/200 in staining buffer. Fluorescence was measured using a FACScan (Becton–Dickinson, Erembodeggem, Belgium).

RESULTS

Identification of a novel gene located adjacent to the gene encoding CLF-1. Sequence analysis of the DNA located 3′ to the gene for CLF-1 (7), within the 40,668-bp cosmid R30292 derived from chromosome 19p12 between the UBA52 gene and the marker D195451, revealed seven putative exons in a reverse orientation to those encoding CLF-1. Six of these putative exons aligned with a number of different ESTs indicating that they were transcribed. The seven exons were subsequently assembled and the resulting sequence was used to query the GenBank database and dbEST. Although the sequence matched with no known cDNA sequences in the GenBank database, 43 ESTs of human origin were identified sharing a high degree of homology with the novel putative cDNA sequence, along with 3 ESTs of mouse origin and 2 of rat origin.

Computer-aided alignment of the 43 human ESTs allowed the assembly of a 1589-bp contig containing a putative ATG start codon (at bp 58) with an upstream kozak consensus sequence (13), a 342-amino-acid ORF and a 3′ poly(A) tail. The ATG start codon was followed by sequence which coded for a putative signal peptide in the same frame (Fig. 1).

The fact that the last predicted exon (exon 7, running from nucleotides 40,023 to 40,140) aligned to nucleo-

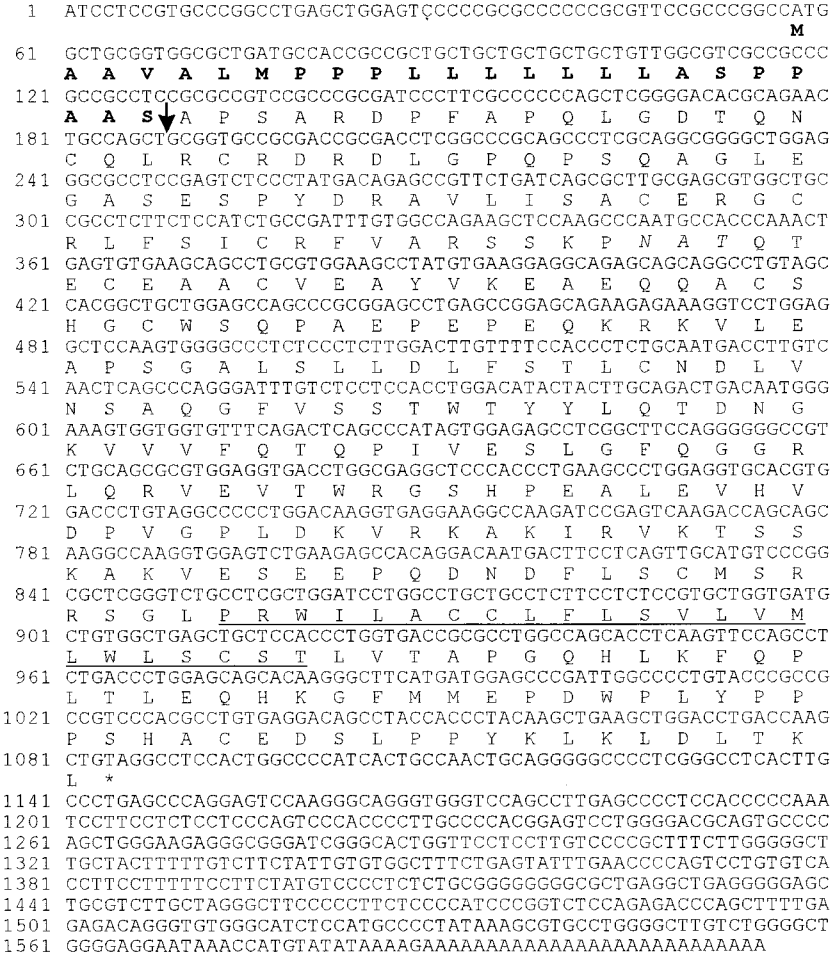

FIG. 1. Nucleotide and deduced amino acid sequence of the BSMAP cDNA. An open reading frame encodes a polypeptide consisting of 342 amino acids. The putative signal peptide is shown in bold with the black arrow indicating the potential cleavage site. The potential N-linked glycosylation site is shown in italic. The putative transmembrane domain is underlined.

tides 612 to 729 of the EST derived cDNA contig suggested that only the partial gene sequence was present in the cosmid R30292. We therefore used the 3′ end of the R30292 cosmid sequence to identify a second cosmid (R30085) overlapping with R30292 at the 3′ end. This additional sequence information allowed us to identify the last exon and therefore the complete gene structure. The novel gene is made up of 8 exons and spans a region of 8137 bp (Fig. 2).

Analysis of the full-length ORF revealed a protein sequence of 342 amino acids with one potential N-linked glycosylation site. Hydrophobic analysis revealed two regions of hydrophobicity, one at the N terminus representing the signal peptide (predicted from amino acids 1 to 24, encoded within exon 1), and a second between amino acids 266 and 288 (encoded within exon 7). The TMpred and TMHMM programs predicted that the latter region is likely to represent a transmembrane domain, and that BSMAP is expressed as a type-I membrane-bound protein. Pattern and motif searching with existing databases using the amino acid sequence gave no indication as to a possible function of the protein, although the sequence did share significant homology with a recently identified protein of unknown function cloned from $CD34^+$ hematopoietic stem/progenitor cells (14), with 37% identity between the two amino acid sequences (Fig. 3).

We also identified three murine and two rat ESTs, with Accession Nos. W85136, AA444173, AI551572, AA942798, and AI412418, respectively, all of which share significant homology with BSMAP both at the nucleic acid and amino acid level. Indeed, the predicted amino acid sequence of the two rat ESTs (AI412418 and AA942798) showed 73 and 81% identity respectively with the BSMAP amino acid sequence, the murine ESTs showing 82% identity for both AA444173 and AI551572 (these two sequences being derived from the same cDNA clone) and 70% identity for W85136. We therefore propose that these ESTs represent cDNAs encoding the mouse and rat homologues of BSMAP.

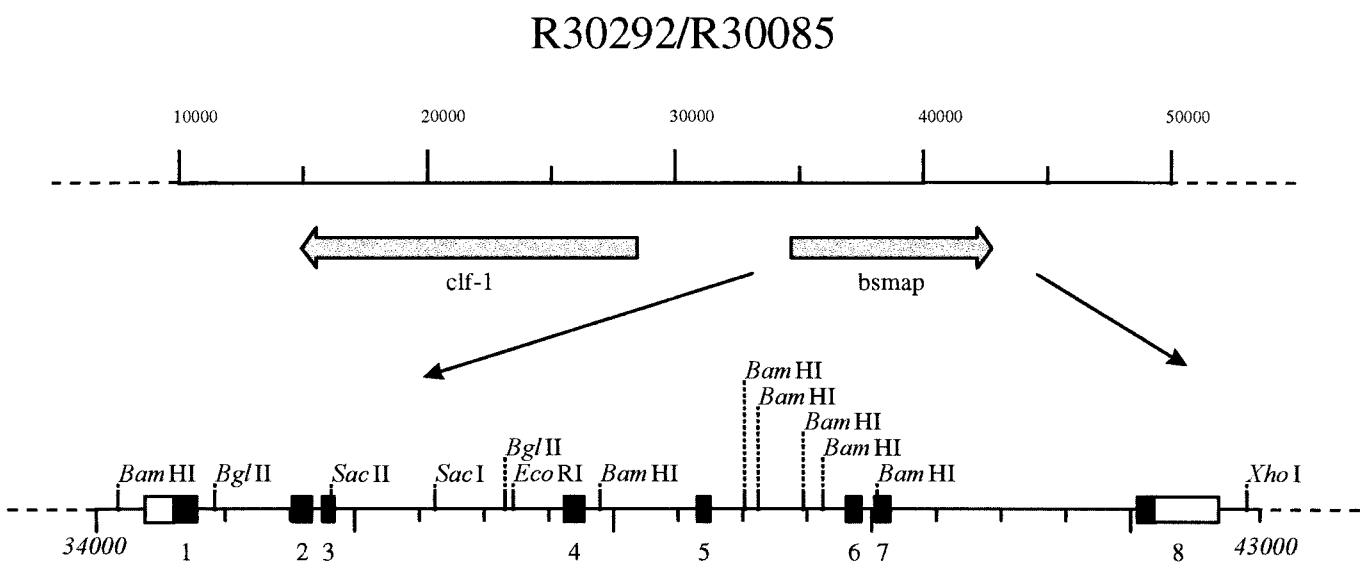

FIG. 2. BSMAP gene structure. The boxes represent exons which are numbered. Filled boxes represent the coding region while open boxes represent the 5′ and 3′ UTR. A partial restriction map covering the region is shown above the exons. The line above represents the assembly of the cosmids R30292 and R30085, with numbers representing the distance in bases from the start of the cosmid R30292. The genes for CLF-1 and BSMAP are represented as arrows.

BSMAP cDNA cloning. We first identified HEK 293 as a cell line expressing detectable levels of BSMAP mRNA by RT-PCR (data not shown). The BSMAP cDNA coding region was subsequently cloned by PCR amplification from HEK 293-derived first strand cDNA using BSMAP-specific synthetic oligonucleotides designed to amplify a PCR product of 1031 bp encoding the full-length ORF, including the hypothetical ATG start codon. A DNA product of a size corresponding to that predicted for the BSMAP cDNA was amplified and subsequently cloned. Sequence analysis confirmed that the derived cDNA sequence contained the full length ORF with no nucleotide mismatches when compared to the cDNA sequence derived from the predicted exon assembly and the BSMAP cDNA assembled from ESTs.

Tissue distribution of BSMAP-specific mRNA. The source of origin of ESTs encoding BSMAP were first examined in order to determine in which tissues BSMAP mRNA was expressed. Of the 48 ESTs identified, 37 were derived from brain, suggesting a significant level of expression of BSMAP-specific mRNA in this tissue. The 3 ESTs derived from human synovial sarcoma, the 3 derived from human fetal tissue, and the 3 derived from mouse embryos also pointed to an expression of BSMAP mRNA in these tissues.

The expression of BSMAP mRNA was analyzed by Northern blotting using polyadenylated RNA derived from twelve different human tissues. Amongst these tissues, a high level of BSMAP-specific mRNA expression was detected in brain, a signal with a molecular

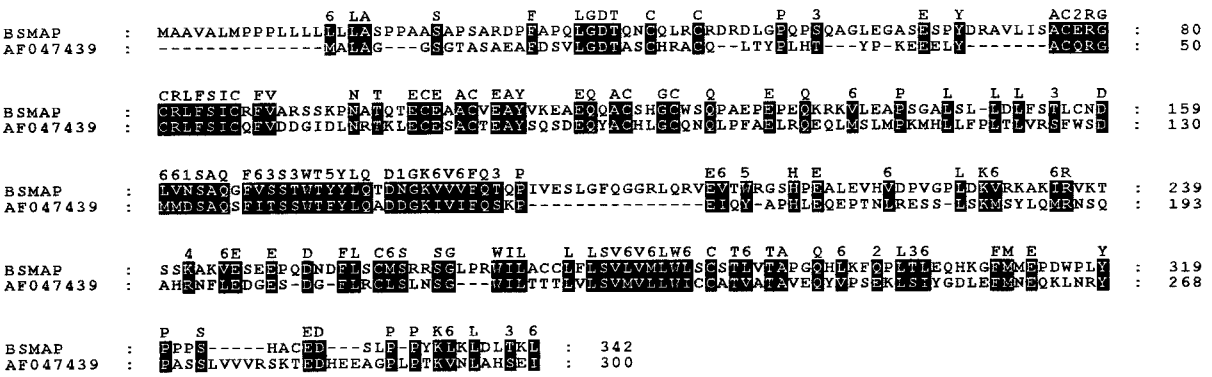

FIG. 3. Alignment of the BSMAP amino acid sequence with the deduced amino acid sequence of the novel cDNA derived from CD34$^+$ hematopoietic progenitors (called by its GenBank accession number) using CLUSTAL W. Residues boxed in black are those showing amino acid similarity or identity, with a consensus sequence shown above the alignment.

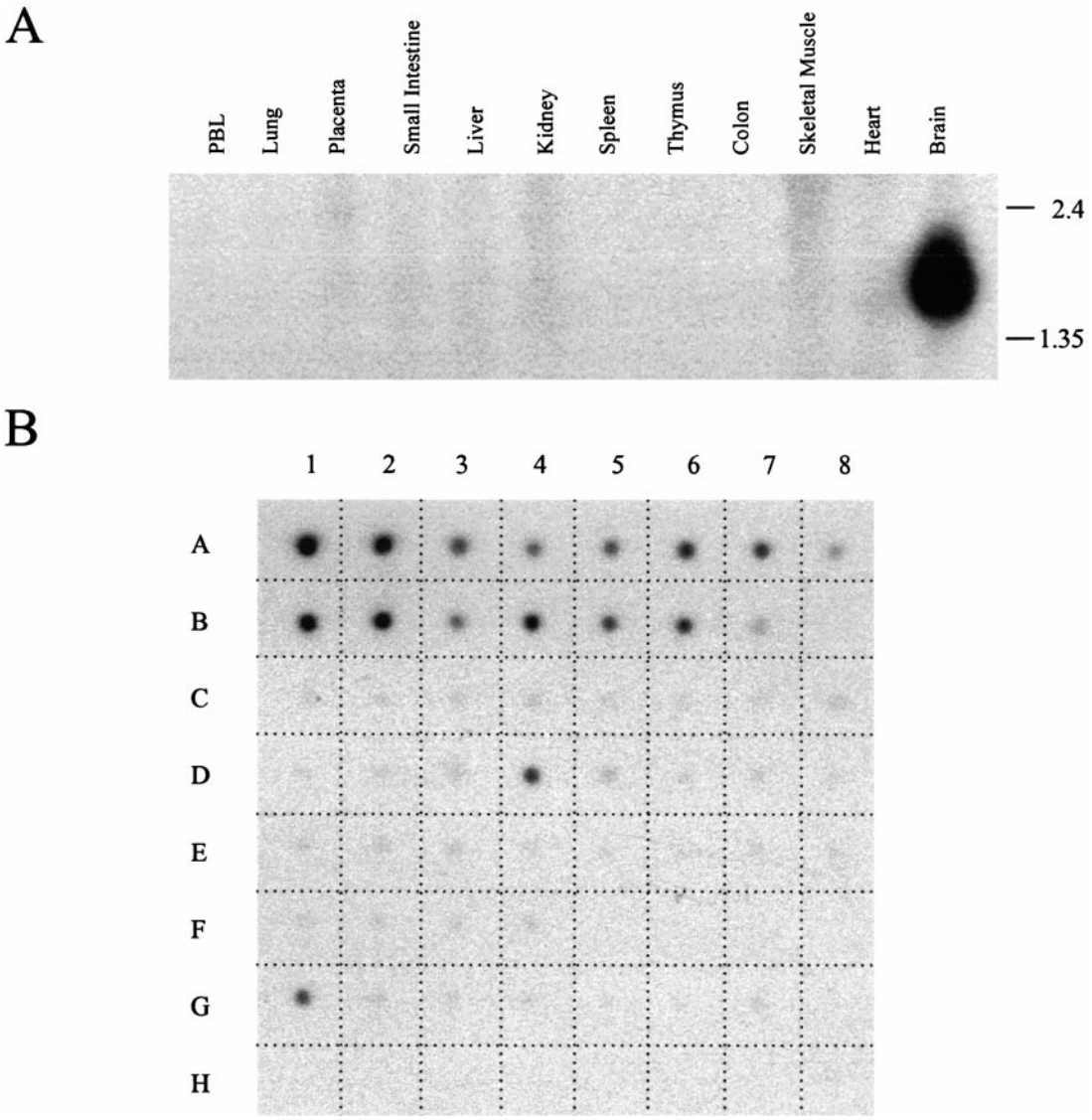

FIG. 4. Northern and Dot blot analysis of BSMAP mRNA tissue distribution. Poly(A)$^+$ mRNA blots were hybridized with a radiolabeled cDNA encoding the full length ORF as a probe. (A) Northern blot containing poly(A)$^+$ RNA derived from 12 different human tissues as indicated. Autoradiography exposure time was 8 hours at −70°C. (B) Dot blot containing poly(A)$^+$ RNA derived from 50 different human tissues. A1, whole brain; A2, amygdala; A3, caudate nucleus; A4, cerebellum; A5, cerebral cortex; A6, frontal lobe; A7, hippocampus; A8, medulla oblongata; B1, occipital lobe; B2, putamen; B3, substantia nigra; B4, temporal lobe; B5, thalamus; B6, nucleus accumbeus; B7, spinal cord; D4, pituitary gland; G1, fetal brain. Autoradiography time was 2 h at room temperature.

size corresponding to that of the cloned cDNA being readily detected following an exposure time of 8 h (Fig. 4A), confirming the above observation that approximately 78% of all BSMAP specific ESTs were derived from this source. Very low levels of expression were also detected in kidney, liver, small intestine and placenta following a prolonged exposure of the hybridized membrane (data not shown). We therefore examined the distribution of BSMAP specific mRNA within the different regions of the CNS using a polyadenylated RNA dot blot. We found a high level of expression of BSMAP RNA in most of the regions of the CNS included as well as in fetal brain and the pituitary gland, relative to the other tissues present on the blot, confirming the observation that BSMAP mRNA is both highly and preferentially expressed in the brain. Strongest expression was seen in whole brain, amygdala, occipital lobe and putamen, with lowest levels of brain-specific expression found in the spinal cord, medulla oblongata and substantia nigra (Fig. 4B).

Expression of BSMAP in transiently transfected COS1 cells. To verify that the cDNA BSMAP was capable of encoding for a protein product, and to determine whether the protein was expressed on the cell surface, cDNAs encoding the full-length ORF with or

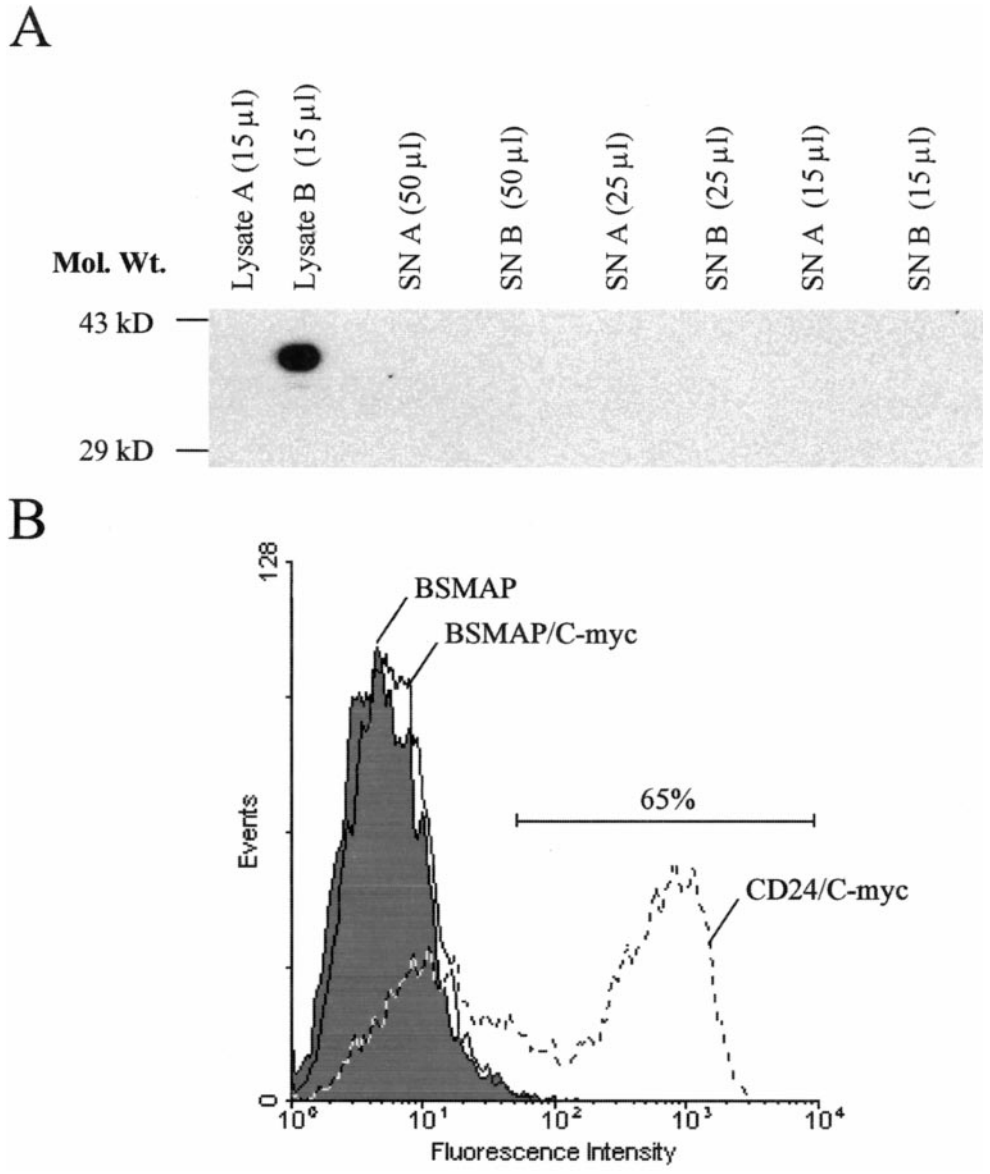

FIG. 5. (A) Detection of BSMAP in transfected COS1 cells by western blotting. Cells were transfected with cDNA constructs encoding BSMAP with or without a C-myc antibody recognition tag as indicated. Proteins from transfected cell lysates (15 μl) and supernatants (volume indicated) were resolved under reducing conditions on a 10% SDS–polyacrylamide gel, electrotransferred to nitrocellulose filters and detected with an anti-C-myc tag mAb. A, BSMAP; B, BSMAP/C-myc. (B) Detection of BSMAP and CD24 on the surface of transfected COS1 cells by flow cytometry. Cells were incubated successively with the anti-C-myc tag mAb and a FITC-conjugated sheep anti-mouse secondary antibody and fluorescence detected by flow cytometry.

without an N-terminal C-myc antibody recognition tag were cloned into the mammalian expression vector pCDNA3 and used to transfect COS1 cells in a transient fashion. Western blot analysis using the anti-C-myc mAb revealed that the cDNA encoded a protein product of approximately 40 kDa, in accordance with the 38-kDa size predicted, which was detectable in the lysate of cells transfected with the cDNA encoding BSMAP containing the C-myc tag. As expected, no band was detected with the lysate of cells transfected with the cDNA encoding the native BSMAP. We also failed to detect a signal in the supernatant of cells transfected with cDNA encoding BSMAP and cells transfected with BSMAP containing the C-myc tag even following prolonged exposure, suggesting (as expected by the protein sequence) that BSMAP was not soluble (Fig. 5A).

Flow cytometry was subsequently used to determine the cellular localization of the protein. As controls, cells were transfected with either the plasmid expressing BSMAP alone (without C-myc tag), or a plasmid expressing the surface protein CD24 with a C-myc tag

contained within the extracellular region (CD24/C-myc). We observed no staining of the surface of cells transfected either with the BSMAP or BSMAP/C-myc expression constructs, contrasting with 65% of CD24/C-myc transfected cells staining positive (Fig. 5B). These results suggest that BSMAP is not expressed on the surface of transfected COS1 cells. Intracellular fluorescent staining was used to verify that BSMAP/C-myc was indeed expressed within the transfected COS1 cells (data not shown).

DISCUSSION

We have identified a gene localized on chromosome 19p12 encoding a novel putative membrane-bound human protein, BSMAP, and cloned the derived cDNA. BSMAP specific mRNA was found to be both highly and preferentially expressed in human brain.

BSMAP shows no significant homology with any known proteins, but does share significant homology with the protein sequence derived from a novel and uncharacterized cDNA recently deposited in GenBank and cloned from $CD34^+$ hematopoietic precursor cells (14). The homology extends throughout both protein sequences including a strong homology within the putative transmembrane domain of BSMAP (Fig. 3). This suggests that this novel $CD34^+$ precursor cell protein could also be a putative membrane-bound protein. A large number of ESTs encoding the $CD34^+$ precursor cell cDNA sequence were identified, having an extremely wide ranging distribution, suggesting a fairly ubiquitous expression pattern (data not shown). It therefore appears, judging by the source of origin of ESTs for BSMAP and the novel $CD34^+$ precursor cell protein, that although the two proteins share a high level of homology, they do not share the same tissue distribution.

BSMAP specific mRNA is preferentially expressed at high levels throughout the CNS and in the pituitary gland, as shown by Northern blotting, dot blotting and the origin of BSMAP specific ESTs. It therefore appears that such a strong and preferential expression of BSMAP mRNA in these tissues suggests a role for the protein in brain function. It is also worth mentioning that the 3 human fetus and 3 mouse embryo ESTs pertaining to BSMAP could point toward a role for this protein during development. *In situ* hybridization studies on mouse embryos would surely be beneficial in elucidating a potential role for this protein in embryonic development.

The extreme 5′ end of the BSMAP cDNA sequence contains a cap consensus and it is therefore likely that it represents a site of transcription initiation. We looked for putative transcription factor binding sites within the sequence directly upstream of this site within the cosmid R30292 (bp 34,000–34,550). Interestingly, a sequence strongly homologous to the cAMP response element (CRE) motif, TGACGTCA, was found at position −36 to −29 from the putative transcription initiation site suggesting that the CRE binding protein (CREB) could be playing a role in the control of expression of BSMAP.

A 40-kDa band was detected in the lysate of COS1 cells transfected with the BSMAP/C-myc expression construct by Western blotting. This corresponds well with the predicted nonglycosylated protein size of 37.6 kDa. It should be noted that BSMAP has only one potential N-linked glycosylation site, thus it is unlikely that glycosylation causes a large increase in the molecular weight of the protein (Fig. 5A). Intracellular fluorescent staining also revealed that the protein was well expressed within the transfected cells with an efficiency of cell transfection greater than 50% (data not shown). The supposed extracellular region of the protein could not however be detected on the outside of the cell by flow cytometry, in contrast with the prediction that BSMAP is a type-I membrane-bound glycoprotein (Fig. 5B), suggesting that the protein could be retained within an organelle or intracellular membrane. We submitted the protein sequence to PSORTII (http://psort.nibb.ac.jp:8800/), a program which predicts protein sorting signals and localization sites. Along with the putative signal peptide and transmembrane domain, the program also predicted the presence of an ER membrane retention signal in the C-terminus of BSMAP (DLTK). It therefore seems plausible that the protein is retained within this subcellular compartment.

Using the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/Omim/), we failed to identify any genetic diseases involving CNS function linked to chromosome 19p12. We also looked in the syntenic region of the mouse genome, situated within chromosome 8, sharing many of the genetic markers (COMP, JUND, MEF 2B, MEL, and JAK 3) found on human chromosome 19p12-13.1 in close proximity to the BSMAP gene. One neurological phenotype, myodystrophy (myd, 15), was identified, the gene responsible for which has yet to be isolated. It has been suggested, however, that the phenotype is actually homologous to the human disorder facioscapulohumeral muscular dystrophy 1A (FSHD1A), localized on chromosome 4q. This is supported by the fact that this particular region of mouse chromosome 8 also contains a segment of homology on human chromosome 4q, the two regions sharing markers such as Ucp, Kal3, Cfl1, and Clc5 (16).

It is likely, however that due to its preferential tissue distribution, BSMAP is a protein playing a role in the regulation of brain function. The elucidation of its cellular distribution within the CNS and embryo through *in situ* hybridization and the generation of mAbs will help to further characterize this

novel protein leading to the elucidation of its physiological function.

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