Expression of a Novel Murine Phospholipase D Homolog Coincides with Late Neuronal Development in the Forebrain*

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Members of the phospholipase D (PLD) superfamily are defined by the conserved HXXXXXXD motif, which is essential for the catalytic function of mammalian PLD. PLD enzymes are thought to play roles in signal transduction and membrane vesicular trafficking in mammalian cells. Here we describe a 54-kDa novel murine polypeptide (designated SAM-9) that is predicted to be a membrane-associated member of the PLD superfamily. SAM-9 shares 40, 30, and 29% amino acid identity with potential orthologs, in vaccinia virus, Caenorhabditis elegans, and Dictyostelium discoideum, respectively, and belongs to a subclass of PLD homologs in which the second HXXXXXXD motif is imperfect and harbors a conserved Asp to Glu substitution. The sam-9 gene has more than eight exons, and the two HXXXXXXD motifs are encoded by two highly conserved exons. The expression of the sam-9 gene is greater in the brain than in non-nervous tissue and appears to be predominantly of neuronal origin. sam-9 expression is pronounced in mature neurons of the forebrain and appears to be turned on at late stages of neurogenesis as revealed by in situ hybridization analysis of sam-9 expression during postnatal development of the hippocampal formation and the primary somatosensory cortex.

The mechanisms underlying migration and postmigratory development of neurons is a central theme in neurobiology. Although various hypotheses have been formulated to explain the region-specific morphogenesis of the telencephalon, the processes controlling forebrain development are largely unknown (1, 2). The earliest murine telencephalic marker, which has been identified to date, is expressed in the prospective telencephalon from embryonic day 8 (3), whereas region-specific markers first appear later (4, 5).

The cells of the cerebral cortex originate in the proliferative neuroepithelium that lines the forebrain vesicle. From this location, immature neurons migrate along the processes of radial glia toward the pial surface to generate the layers of the cortex (6). The first post-mitotic neurons form the preplate, which is later split by the cortical plate that is generated subsequently by the invasion of true cortical neurons (7). The latter neurons are distributed in an inside-out sequence to create layers VI to II of the cortex, which are sandwiched between the superficial and deep components of the original preplate (i.e. the marginal zone (layer I) and the subplate) (8). In layer IV of the primary somatosensory (“barrel”) cortex of rodents, a whisker-related pattern is apparent where thalamocortical projection neurons form cellular aggregations (designated barrels) that have a 1:1 relationship with a particular whisker on the face (9). Individual barrels consist of a ring of densely packed neurons arranged in a cylindrical array around a hollow center of low cell density. In mice, formation and maturation of individual barrels in layer IV occur postnatally and proceed well into the 2nd week of extrauterine life (10).

In the stratum pyramidale of the hippocampus, postmigratory neurons are laid down in an “inside-out” fashion, that is the first neurons to form are those located in the deeper parts of the mature layer, and the more superficial cells are those that form progressively later (11, 12). Generation of pyramidal neurons in the stratum pyramidale occurs at different times along a subicular-to-dentate morphogenetic gradient resulting in deposition of CA1 neurons prior to CA3 neurons (12). Formation of the fascia dentata in rodents is mainly a postnatal process (13), and deposition of mature granule neurons in the limbs of the dentate gyrus begins in the suprapyramidal limb and proceeds into the infrapyramidal limb, establishing the suprapyramidal-to-infrapyramidal morphogenetic gradient. In addition, an “outside-in” gradient is also apparent, in which cells destined for the superficial layers are generated prior to cells in the deeper layers (11, 13).

When neurons reach their final destination within the brain, a series of complex cellular processes take place including axonal outgrowth, target innervation, and fine-tuning of axonal branches and synapses (14, 15). During this terminal differentiation, new sets of genes are turned on, and others are switched off. Here we describe the cloning, expression, and characterization of a novel highly conserved gene, designated sam-9, which seems to be turned on late in neuronal development as revealed by its developmentally regulated expression in the hippocampal formation and the primary somatosensory cortex. The SAM-9 ORF1 harbors two highly conserved stretches that comprise an HXXXXXXD motif characteristic for proteins of the phospholipase D (PLD) superfamily (16–18) and exhibits extensive amino acid identity with putative PLD orthologs in vaccinia virus, Caenorhabditis elegans, and Dictyostelium discoideum. Because mammalian PLD homologs have...
been proposed to mediate phospholipid signal transduction in various cell contexts (reviewed in Refs. 19–21), SAM-9 may play a role in intracellular signal transduction in mature neurons.

**EXPERIMENTAL PROCEDURES**

**RNA Isolation and Northern Blot Analysis.** Total RNA was prepared by CsCl gradient ultracentrifugation of guanidine isothiocyanate-lysed cells. Poly(A\(^+\)) RNA was isolated from total RNA using oligo(dT)-cellulose-spun columns (Pharmacia Biotech, Sweden). For Northern blotting, RNA was purified from various organs and electrophoresed in formaldehyde/agarose gels, blotted onto Hybond-N membranes.

**Library Screening, DNA Sequencing, and in Vitro Coupled Transcription/Translation.**

Poly(A\(^+\)) RNA (3 \(\mu\)g) obtained from 6E12 cells (22) was used to construct an oligo(dT)-primed cDNA library with the SuperScript Choice System (Life Technologies, Inc.). A total of 100 ng of cDNA, comprising the 1–5-kb fraction, was ligated to lambda Ziploc EcoRI arms (Life Technologies, Inc.), and phage clones were obtained following in vitro packaging (Gigapack III Gold Packaging Extract, Stratagene, La Jolla, CA) and infection of Escherichia coli strain Y 1090ZL. Phage clones were screened by hybridization of colony lifts to subtracted cDNA probes prepared as described below. Excision of pZL1 plasmid clones was carried out by phage infection of the excision strain DH10B-Zip (Life Technologies, Inc.).

First strand cDNA were prepared from 3 \(\mu\)g of poly(A\(^+\)) RNA obtained from the semi-differentiated Schwannoma cell line 56-24 (23) using oligo(dT) as a primer and Moloney murine leukemia virus SuperScript II as described (24). Subtracted 56-24-enriched probes were prepared by hybridizing, for 22 h, 400 ng of target cDNA to 7.5 \(\mu\)g of poly(A\(^+\)) RNA obtained from the undifferentiated Schwannoma cell line 64-39 (22) and subjected to chemical cross-linking with 2,5-diaziridinyl-1,4-benzoquinone (kindly provided by Amersham Corp.), and labeling as described previously (24).

A SAM-9 genomic clone was isolated by screening 1 \(\times\) 10\(^6\) clones of a mouse genomic DNA library in lambda Fix II (Stratagene, La Jolla, CA), using the SAM-9 cDNA as a probe. DNA sequencing was performed using the dye terminator method (Applied Biosystems, Foster City, CA) with a DNA thermal cycler (Perkin-Elmer Cetus) and an automated sequencer (model 373A, Applied Biosystems).

**In vitro transcription/translation of the SAM-9 cDNA was performed according to the manufacturer’s specifications, using the TNT-coupled reticulocyte lysate system (Promega) and translational grade [\(^{\text{35}}\)S]methionine (Amersham Corp.).**

**In Situ Hybridization.**

**Animals—**Newborn postnatal day 0 (P0), P7, and P14 and young adult (30 g) NMRI mice (Biomedical Laboratory, Odense University) were either decapitated (P0 animals) or deeply anesthetized by intraperitoneal injection with sodium pentobarbital (P7, P14, and adult animals), and the brains (P0, P7, and P14 animals) and the spinal cord, lumbar dorsal root ganglion, and sciatic nerve (adult animals) were removed and sectioned into a parallel series of 16- \(\mu\)m thick cryostat sections. One series of sections was mounted on gelatin-coated glass slides and stained with toluidine blue to show the general histological pattern. The remaining sections were mounted on RNAse-free Superfrost Plus glass slides and stored in sealed boxes at \(-20^\circ\)C until hybridized.

**Probes—**In situ hybridizations were performed using alkaline phosphatase-labeled 30-mer antisense SAM-9 oligonucleotide probes (5’-AGGCCACCTTCCAGTCCTGGTACATCGGTTT-3’ and 5’-ATGGG-AAACCCGACCCCTCGAGATGCT-3’ corresponding to nts 426–456 and nts 678–708, respectively. A 27-mer antisense probe (5’-XCTTGCT-C TTCACCACCTTGCAGATGCT-3’) corresponding to nts 833–859 of the SAM-9 cDNA, was included in the positive controls. All probes were purchased from DNA Technology (Aarhus, Denmark).

**Procedure—**One day prior to hybridization, the frozen slides were dried for 10 min at 55 \(^\circ\)C, immersed in 96% ethanol at 4 \(^\circ\)C overnight, air-dried, and hybridized with 5–10 \times 10\(^{-12}\) mol of probe per ml of hybridization buffer (50% formamide, 4 \% sodium sodium chloride (SSC), 0.04 g of polyvinylpyrrolidone, 0.04 g of bovine serum albumin, 10% dextran sulfate, and 500 g of single-stranded salmon sperm DNA per ml). The sections were hybridized overnight at 37 \(^\circ\)C and rinsed in 1× SSC for 3 × 30 min at 55 \(^\circ\)C followed by 2 × 15 min in Tris-HCl, pH 9.5, and subjected to alkaline phosphatase development using nitro blue tetrazolium (Sigma, UK) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, UK) as substrates. Development of the reaction product was stopped by immersing the slides in water. The sections were covered-slipped with Aquamount (Merck, Darmstadt, Germany).

**Controls—**Although the in situ hybridizations presented here were obtained using the SAM-9 probe spanning nts 426–456, sections hybridized with either of the two SAM-9 probes displayed similar cellular, spatial, and temporal hybridization signals. Sections hybridized simultaneously with both probes yielded stronger hybridization signals than sections hybridized with either of the two probes alone. Sections hybridized with the hybridization buffer alone, with an excess of unlabeled probe (100–200 \(\times\)), or hybridized subsequently to treatment with ribonuclease A (50 \(\mu\)g/ml; Pharmacia, Denmark), revealed no specific cell staining. Hybridization with the glyceraldehyde-3-phosphate dehydrogenase probe revealed pronounced hybridization signals in neurons.

**RESULTS**

**Cloning of the SAM-9 cDNA—**To identify mRNAs expressed in differentiated Schwann cell tumors, we employed a subtractive cloning technique based on chemical cross-linking subtraction (24). Single-stranded cDNA was synthesized from poly(A\(^+\)) RNA purified from the semi-differentiated Schwannoma cell line 56-24, and subtracted with a 19-fold excess of poly(A\(^+\)) RNA from undifferentiated Schwannoma cells of line 64-39 to generate the subtracted single-stranded cDNA hybridization probe. Approximately 100,000 clones, from a non-amplified mouse oligodendrocyte cDNA library, were screened by hybridization to the subtracted probe. From the primary screening, 40 clones were pooled and rehybridized to the same probe. Of these, 17 clones hybridized in a reproducible manner to the subtracted probe. Hybridization probes were prepared from these clones and used in Northern blots containing RNA from 56-24 and from 64-39 cell lines. Of the 17 clones, designated SAM 1 to 17 (for Schwannoma-associated mRNA), one was found to be highly up-regulated in 56–24 cells, whereas the remaining clones were up-regulated to a lesser degree (data not shown). In this report we have characterized a 2.3-kb clone, designated SAM-9 (GenBank\textsuperscript{TM} accession number AF026124), which gave a strong hybridization signal to RNA purified from the brains of adult mice but not to RNA purified from non-nervous tissues, including kidney, spleen, liver, testes, lung, heart, and ovary (Fig. 1). A weak hybridization to RNA purified from day 8 to 9 mouse embryos was observed, and only moderate levels of sam-9 expression occurred in the peripheral nervous system and kidney tissues (Fig. 1).

\(^{2}\) K. M. Pedersen, B. Finsen, J. E. Celis, and N. A. Jensen, manuscript in preparation.
Structural Analysis of the SAM-9 cDNA—The SAM-9 clone appeared to contain near full-length cDNA, estimated to be approximately 2–2.5 kb on the basis of Northern blot analysis. The SAM-9 cDNA is 2245 base pairs long and contains one long ORF encoding a deduced protein of 488 aa, which starts with the first methionine codon at nts 417–420 and ends in an in frame termination codon at nts 1882–1885 (Fig. 2). The deduced 488-aa sequence has a predicted molecular weight of 54,388 and a pI of 6.51. Given that the SAM-9 cDNA is full-length, the mRNA consists of a 5' untranslated region of 416 nts and a 3' untranslated tail of approximately 360 nts.

Between aa 54 and 488, the SAM-9 translation product is 94% identical to Hu-k4 (26), which encodes the human homolog of SAM-9. However, the first methionine of the SAM-9 ORF is positioned 53 aa upstream of the first methionine codon of the Hu-k4 ORF, and comparison of the DNA sequence spanning this region revealed a stop codon at nt 407 in the Hu-k4 sequence. Sequencing of the entire plus and minus strands of the SAM-9 cDNA, as well as repeated sequencing of the region corresponding to this mismatch in the human sequence, confirmed that the first methionine codon is located at nt 417–420 in the SAM-9 ORF. The estimated molecular masses of SAM-9 and Hu-k4 are 54 and 48 kDa, respectively, and in vitro transcription-coupled translation of the SAM-9 cDNA revealed a single major translation product of approximately 54 kDa (Fig. 3), which is consistent with the predicted molecular weight of the SAM-9 ORF.

By using a motif search program (ProSearch Software; L. Kolakowski), putative post-translational modification sites were identified in the SAM-9 ORF, including asparagine glycosylation sites at Asn97, Asn132, Asn234, Asn385, and Asn430 and putative protein kinase C phosphorylation sites at Ser 300, Ser318, and Ser 371, and at Thr 420. Casein kinase II sites appeared in Ser88, Ser118, Thr130, Thr275, Thr387, and Ser456. Two putative tyrosine kinase sites appeared at Tyr271 and at Tyr470, and a CaaX motif encoding a putative prenylation site at Cys485.
The SAM-9 ORF shares 40% amino acid identity with the K4L protein of vaccinia virus (29), 29% identity with an ORF, designated DDI1093A cds3 on the genome of *D. discoideum* (30), and 30% amino acid identity with the aminoterminal region of a large ORF, designated CELT05C3 cds2 (31) on chromosome V of *C. elegans* (Fig. 4A). In view of the conserved amino acid changes, the similarity between the various ORFs is even greater (Fig. 4A). Two stretches on the SAM-9 ORF, which comprise the two HXXXXXD motifs, displayed high overall amino acid similarities with the various PLD homologs, in the order of 61 and 48%, respectively (Fig. 4B), suggesting the existence of common structural and catalytic domains in these proteins. Hydrophobicity plots (PEP-ALLWINDOW) showed that the ORFs harbored similar hydrophathy profiles (data not shown). Two other PLD homologs, the vaccinia virus major envelope antigen VP37 and the *C. elegans* ORF CELE04F6 cds1, displayed moderate (in the order of 20–23%) amino acid homology to SAM-9, indicating that they are distant members of the *sam-9* gene family.

Cloning of the *sam-9* Gene—A mouse genomic clone was isolated from a λ library by screening with the SAM-9 cDNA. Restriction mapping revealed that most of the hybridization signal corresponded to two Xba-1 fragments that were subcloned and partially sequenced. Eight exons encoding aa 83–488 of SAM-9 were located in these Xba-1 fragments. Approximately 666 nts, corresponding to the 5’ end of the cDNA, were not included in the λ clone. A composite map of the *sam-9* gene is shown in Fig. 5A, in which the exons have been arbitrarily designated 1–8. As seen in Fig. 5B, exon 3, which encodes aa 182–224, and exon 7, which encodes aa 394–426, display the highest levels of evolutionary conservation. Notably, amino acids encoded by exon 3 are 100% identical with those of Hu-k4 ORF, 67% identical with K4L, and about 46% identical with the Hu-k4, K4L, DDI1093A cds3, and CELT05C3 cds2 ORFs. Exon 7 was 91, 58, and 36% identical to these ORFs, respectively, strongly suggesting that the two exons are essential for *sam-9* gene function. Notably, the HXXXXXXD motif, spanning aa 199–214, is located within exon 3, and 13 out of 16 aa in the HXXXXXXD motif, spanning aa 414–429, are encoded by exon 7.

Expression of the *sam-9* Gene in Vivo—Northern tissue blots showed that the *sam-9* mRNA is up-regulated in the postnatal brain relative to non-nervous tissues and that early mouse embryos expressed low levels of *SAM-9* (Fig. 1).

*In situ* hybridization with synthetic oligonucleotide probes was used to determine the expression pattern of the *sam-9* gene in the intact postnatal central nervous system. In the adult central nervous system, a spinal-to-telencephalic expression gradient was apparent with the strongest general hybridization signal present in telencephalic regions, including neocortex, entorhinal cortex, hippocampal formation, striatum, and septum, whereas moderate to low signals were observed in the olfactory bulb, thalamus, mesencephalon, cerebellum, and spinal cord (Figs. 6A and 7, A–D). In addition, a moderate hybridization signal was observed in the cell bodies of dorsal root ganglion neurons but not in fiber bundles of the sciatic nerve (Fig. 7, E and F). In both the central nervous system and the peripheral nervous system, the hybridization signal was almost exclusively of neuronal origin, whereas neuroglia and Schwann cells appeared to express low to undetectable levels of *SAM-9* mRNA.

*sam-9* expression follows a caudal-to-rostral developmental gradient, in which hybridization signals were present in the mes- and diencephalon at birth and became progressively more pronounced in the telencephalon during postnatal development (Fig. 6, A–D). In P0 animals, *SAM-9* hybridization signals appeared in mesencephalon, diencephalon, Ammon’s horn, and entorhinal cortex (Fig. 6D). In P7 animals, *sam-9* expression was pronounced in neurons of the anteroventral thalamic nuclei, in septum, striatum, hippocampal formation, and layer V pyramidal neurons of parietal neocortex (Fig. 6C). The overall expression pattern of *sam-9* in P14 animals was similar to that of P7 animals, with the exception that the hybridization signal was generally stronger in telencephalic regions than it was in diencephalic and mesencephalic regions (Fig. 6, B and C). In the adult, the strongest general hybridization signals were obtained in telencephalic regions, notably in neurons associated with the perforant path of the hippocampal formation,
including layer II neurons of the entorhinal cortex, hilar and granule neurons of the fascia dentata, and neurons in the stratum pyramidale of Ammon's horn (Figs. 6A and 8D).

Because neurogenesis in the murine fascia dentata is essentially a postnatal process, we investigated whether or not sam-9 expression was developmentally regulated in this structure. During late embryonic and early postnatal development, granule neurons settle in the limbs of the dentate gyrus along

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**Fig. 4.** Comparison of the SAM-9 protein sequence with putative PLD orthologs in homo sapiens, vaccinia virus, D. discoideum, and C. elegans. A, amino acids are designated by one-letter code; identical amino acids are connected by black boxes; similar amino acids are indicated by gray boxes. SAM-9, mouse; Hu-K4, human; K4L, vaccinia virus; DDI1093a, D. discoideum; CELT05C3, C. elegans. B, a schematic representation of evolutionarily conserved domains in the SAM-9 polypeptide. Two regions on SAM-9 shared high amino acid identity (61 and 48%) with Hu-K4, K4L, DDI1093a, and CELT05C3 ORFs. The numbers refer to the amino acid residues at the beginning and end of each of the designated domains. The positions of the HXKXXXXD motifs, characteristic for PLD homologs, are indicated.
the suprapyramidal-to-infrapyramidal morphogenic trajectory (11, 12). In the dentate gyrus of P0 animals, sam-9 expression was pronounced in a thin layer of granule cells located on the outer edge of the suprapyramidal limb but not in granule cells of the infrapyramidal limb or in granule cells of the inner layers of the fascia dentata (“outside-in gradient”) (Fig. 8A). In addition, at this time an inside-out sam-9 expression gradient was apparent in stratum pyramidale of Ammon’s horn, where the strongest hybridization signal occurred in the deepest (earliest settled) neuronal layers. In P7 animals, sam-9 expression was pronounced in both limbs of the dentate gyrus, as well as hilar neurons, and the suprapyramidal-to-infrapyramidal sam-9 expression gradient was still apparent, with the strongest hybridization signal in the outer layer of the suprapyramidal limb (Fig. 8B). The outside-in sam-9 expression gradient was apparent at P7 showing pronounced hybridization to the outer layer granule cells, whereas the inside-out sam-9 expression gradient, observed in the stratum pyramidale of the Ammon’s horn in P0 animals, was less pronounced in P7 animals (Fig. 8, A and B). In P14 animals, the suprapyramidal-to-infrapyramidal expression gradient was no longer obvious, in contrast to the outside-in sam-9 expression gradient that was still apparent (Fig. 8C). There was no evidence of sam-9 expression gradients in the adult hippocampal formation, in which the majority of pyramidal and granule neurons appeared to express high levels of SAM-9 (Fig. 8D).

Because the barrel (i.e. somatosensory) cortex in the mouse begins to assume its adult mature form from P4 onward (10), we also investigated the pattern of sam-9 expression in this cortical area of P0, P7, and P14 animals (Fig. 9). In P0 animals, sam-9 expression was low in neurons of the cortical plate and layer V, whereas moderate levels of sam-9 expression appeared in layer VI neurons (Fig. 9A). At P7, an inside-out expression gradient appeared in which moderate levels of SAM-9 were observed in layer V neurons, and low levels of sam-9 expression appeared in upper layers (i.e. layers IV and III) (Fig. 9B). At this time of development, sam-9 expression in layer IV neurons appeared to be low, and individual barrels exhibited a diffuse morphology (Fig. 9B). On the contrary, sam-9 expression in cells of layers V to III was pronounced in P14 animals. In layer

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**FIG. 5. SAM-9 genomic organization.** A. a partial map of the SAM-9 genomic DNA showing the location of 8 exons. The regions that have been sequenced are indicated with a dotted line. B, the polypeptide sequence encoded by individual exons was compared with those of the indicated homologs. The exons have been arbitrarily assigned 1–8, and the numbers refer to the amino acid residues at the beginning and end of each of these exons.
IV, barrels were clearly discernible as periodicities in the density of sam-9 expressing cells in the wall (i.e. the densely packed cells that form a ring around each barrel) and the less cell dense center of individual barrels (Fig. 9C).

**DISCUSSION**

The chain of events that regulates neuronal development within the brain is still unclear. A prerequisite for unraveling the order of events is the identification of signal transduction genes that are expressed in neurons at various developmental stages. In this report, we show that the expression of a novel murine PLD homolog, designated sam-9, appears to coincide with late neuronal development in the hippocampus and the primary somatosensory cortex. In the dentate gyrus of the hippocampus, the appearance of SAM-9-positive granule cells followed a suprapyramidal to infrapyramidal morphogenic gradient, in which sam-9 expression appeared in the first granule neurons to undergo postmigratory development in the suprapyramidal limb and progressed toward the neurons that developed later in the infrapyramidal limb and in the inner layers of the dentate gyrus. sam-9 expression also appeared to coincide with postmigratory development of pyramidal neurons in the

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**Fig. 6. Localization of SAM-9 mRNA in the brain.** Horizontal sections of mouse brains of adult (A), P14 (B), P7 (C), and P0 (D) mice were subjected to in situ hybridization with an alkaline phosphatase-labeled probe complementary to SAM-9 mRNA. During postnatal development the SAM-9 hybridization signal became progressively more pronounced in telencephalic regions including the neocortex (CTX), striatum (STR), septum (S), and the hippocampal formation (HF). Note the prominent SAM-9 hybridization signals in neurons of the perforant path including the pyramidal (p) and granule (g) neurons of the Ammon’s horn and dentate gyrus and layer II neurons of the entorhinal area (EA) (A). Cortical layers are given in Roman numerals; atn, anteroventral thalamic nucleus; cc, corpus callosum; CER, cerebellum; cp, cortical plate; MES, mesencephalon; mz, marginal zone; OL, olfactory bulb; TH, thalamus. Scale bars, 2000 μm.
Ammon's horn, in that the expression was first detected in the deepest (early developed) layers of the stratum pyramidale and followed an inside-out pattern characteristic for development of postmigratory neurons in this region. Expression of sam-9 in the somatosensory cortex appeared also to occur along an inside-out developmental sequence, in which SAM-9 hybridization signals were more pronounced in deeper layers early in development and became more pronounced in outer layers as development proceeded. In layer IV, sam-9 expression was pronounced in barrels of P14 animals but not in barrels of P7 animals, which supports the notion that sam-9 expression coincides with late neuronal development.

In addition to its expression in telencephalic neurons, sam-9 expression was also detected in the brain stem, thalamus, and dorsal root ganglion neurons, but the levels of expression were conspicuously low in the spinal cord and cerebellum. Moreover, during postnatal development sam-9 expression appeared generally higher in telencephalic regions than in other brain regions. The origin of sam-9 expression was mainly neuronal, with low to undetectable levels of expression in glial cells. The observation that sam-9 expression appeared to be turned on in mature neurons argues against a housekeeping function for the gene in neurons. Furthermore, sam-9 expression in layer II neurons of the entorhinal cortex appeared to be more pronounced in the adult than during early postnatal development. The main projection of the entorhinal cortex to the hippocampus is the perforant path, and efferents of layer II entorhinal neurons terminate mainly on cells in the fascia dentata. Since entorhinal neurons are produced prior to those of the dentate gyrus and their efferents appear to reach the dentate gyrus before the majority of granule cells are generated, perforant path axons from the entorhinal cortex await the genesis and maturation of their target granule cells in the dentate gyrus (32, 33). The pronounced expression of sam-9 in entorhinal
layer II neurons following the formation of the dentate gyrus suggests that SAM-9 is involved in processes associated with target cell innervation, neurotransmission, and neuronal survival but is less involved in early developmental processes, such as axonal growth, neurogenesis, and neuronal migration.

The structure of SAM-9 provides some clues about its function. The presence of a putative carboxyl-terminal prenylation site, an amino-terminal myristoylation site, a amino-terminal HXXKXXXXD motif, and a carboxyl-terminal HXXKXXXXE motif suggests that SAM-9 is a membrane-associated protein of the PLD superfamily (17, 28, 34). Extensive amino acid homology to PLD homologs in poxvirus (K4L), Homo sapiens (Hu-k4), C. elegans (CELT05C3 cds2), and D. discoideum (DDI1093A cds3) was observed, implying that SAM-9 orthologs exist in cells from species as diverse as mammals, nematodes, and amoebae. An interesting feature of these PLD homologs, with the exception
conserved, carboxyl-terminal HXXXXXE motif. This combination is not observed in other known members of the PLD superfamily (17), and the catalytic significance of this feature awaits further characterization. Mutagenesis analyses have shown that the catalytic activity of PLD1 requires two intact HXXXXXD motifs as well as a highly conserved serine/threonine residue in the carboxyl-terminal motif and that a PLD mutant protein harboring a conserved Asp to Glu substitution in the carboxyl-terminal HXXXXXD motif is non-functional (34). Although K4L harbors two consensus HXXXXXD motifs, the protein did not appear to exhibit PLD activity (34), and K4L seems to be less related to PLD than the bacterial phospholipid synthesis enzymes, cardiolipin synthase, and phosphatidylserine synthase (18). We have not been successful in detecting PLD activity with SAM-9 in a simple PLD assay (data not shown). Taken together, these observations suggest that SAM-9 family proteins does something else than cleave phosphatidylcholine. Our findings, however, that the two HXXXXXD motifs are located within two highly conserved regions on the SAM-9 ORF (61 and 48% amino acid identity, respectively, between family members) as well as encoded by two highly conserved exons of the sam-9 gene strongly suggest that the HXXXXXD motifs are important for SAM-9 function. In addition, the SAM-9 ORF (54 kDa) contains an additional 53 amino acids in the amino-terminal region as compared with its human counterpart Hu-K4 (48 kDa). Although we cannot exclude the possibility of a "short" and "long" form of SAM-9 and Hu-K4, it is likely that the Hu-K4 ORFs arise from a frameshift mutation just upstream of the ATG codon in the human clone. The human EST sequences available for this region report 6 different sequences, in which some are as originally reported (26) and others are as found in this report. In vitro coupled transcription/translation of the SAM-9 cDNA revealed a major protein with a molecular mass of approximately 54 kDa, which is consistent with the molecular mass of the SAM-9 ORF.

The genome of poxvirus appears to comprise several genes of vertebrate origin that interact with host-encoded regulatory proteins, presumably to circumvent host defenses better (reviewed in Ref. 35). The VP37 protein contains a single partially conserved HXXXXXD motif and is essential for efficient cell-to-cell spreading by vaccinia virus (36). The vp37 gene is distantly related to sam-9 and to other members of the sam-9 family (data not shown). Although VP37 does not exhibit PLD activity, the partially conserved HXXXXXD motif was shown to be required for efficient cell-to-cell spreading of vaccinia virus (34). The sam-9 family member K4L appears to be non-essential for the replication of vaccinia virus in vitro, and the protein cannot functionally rescue the loss of VP37 activity in virus-infected cell cultures (36). Moreover, some poxvirus either lack a K4L ORF (37) or appear to harbor a truncated version of the gene (26).

Both PLD and phospholipase C (PLC) enzymes have been implicated in signal transduction pathways associated with cell growth and membrane trafficking in mammalian cells (19–21, 38). PLD activity has been detected in D. discoideum during the final stage of development prior to the formation of the "fruiting body" (39). Although little is known about the physiological role of PLD during Dictyostelium development, the slime mold does harbor a single PLC gene, which structurally resembles mammalian PLC-δ (40). In Dictyostelium, expression of PLC occurs throughout development, from aggregation to the fruiting body stage (reviewed in Ref. 41), and despite the multitude of roles that have been attributed to PLC activation in mammalian cells (21), neither developmental nor biochemical phenotypes appeared in Dictyostelium mutants that lacked PLC activity (42). Because there is no evidence of the presence

FIG. 9. Localization of SAM-9 mRNA during postnatal development of the primary somatosensory cortex. A, at P0, the somatosensory cortex consists of layers VI, V, the cortical plate (CP), and the marginal zone. sam-9 expression signals appeared to be most prominent in neurons of layer VI, whereas neurons in layer V and CP displayed low hybridization intensity. B, the cortex of P7 animals consists of layers VI–II, and a sam-9 expression gradient is apparent at this time of development. A pronounced SAM-9 hybridization signal occurred in layer V neurons but not in neurons of upper layers, including the developing barrels in layer IV (arrows). C, at P14, sam-9 expression was pronounced throughout the somatosensory cortex, including neurons of the barrel field in layer IV. Individual barrels appear as differences in cell density within layer IV (arrows). Scale bars, 100 μm.

of K4L that harbors two consensus HXXXXXD repeats, is that the second HXXXXXD motif is imperfect and comprises a conserved amino-terminal HXXXXXD motif and a truncated, but
of other plc genes in *Dictyostelium* (38, 40), the lack of a phenotype in the PLC mutant may be attributed to a functional back-up system in phospholipid signaling, perhaps by expression of PLD homologs. In addition, yeast also appears to harbor not only a single 3-type plc. However, disruption of this gene by gene targeting resulted in viable mutants, which showed increased sensitivity to various stress factors (43). As opposed to the subtle effects of PLC knock-outs in lower eukaryotes, the development of spontaneous seizures and the loss of hilar neurons in the hippocampus (47). The cloning of the *sam-9* gene provides an opportunity to inactivate the gene by homologous recombination. This could provide definitive evidence regarding the role of this gene product in neuronal function, learning, and memory, as well as its contribution to brain dysfunction.

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