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The Limbic System–Associated Membrane Protein Is an Ig Superfamily Member That Mediates Selective Neuronal Growth and Axon Targeting

Aurea F. Pimenta,* Victoria Zhukareva,*
Mary F. Barbe,† Blesilda S. Reinoso,*
Christopher Grimley,‡ William Henzel,‡
Itzhak Fischer,§ and Pat Levitt*
*Department of Neuroscience and Cell Biology
University of Medicine and Dentistry of New Jersey
Robert Wood Johnson Medical School
Piscataway, New Jersey 08854
†Department of Physical Therapy
Temple University School of Allied Health
Philadelphia, Pennsylvania 19140
‡Protein Chemistry Department
Genentech, Incorporated
South San Francisco, California 99432
§Department of Anatomy and Neurobiology
Medical College of Pennsylvania
Philadelphia, Pennsylvania 19129

Summary

The formation of brain circuits requires molecular recognition between functionally related neurons. We report the cloning of a molecule that participates in these interactions. The limbic system–associated membrane protein (LAMP) is an immunoglobulin (Ig) superfamily member with 3 Ig domains and a glycosyl-phosphatidylinositol anchor. In the developing forebrain, *lamp* is expressed mostly by neurons comprising limbic-associated cortical and subcortical regions that function in cognition, emotion, memory, and learning. The unique distribution of LAMP reflects its functional specificity. LAMP-transfected cells selectively facilitate neurite outgrowth of primary limbic neurons. Most striking, administration of anti-LAMP in vivo results in abnormal growth of the mossy fiber projection from developing granule neurons in the dentate gyrus of the hippocampal formation, suggesting that LAMP is essential for proper targeting of this pathway. Rather than being a general guidance cue, LAMP likely serves as a recognition molecule for the formation of limbic connections.

Introduction

Activity independent and dependent mechanisms underlie the generation of precise neuronal connections (Goodman and Shatz, 1993). The former is probably mediated by common recognition strategies utilized by all organisms (Hynes and Lander, 1992; Jessell and Melton, 1992; Goodman, 1994), with guidance cues provided by adhesion molecules operating in combination with diffusible chemotropic factors released from restricted populations of guiding or target cells and molecules providing repulsive cues (Tessier-Lavigne, 1994). Ubiquitously expressed members of the immunoglobulin superfamily (IgSF), integrins and cadherins, can modulate growth of all types of neurons (Jessell, 1988), but it is thought that those molecules expressed uniquely among groups of functionally related neurons are more likely to provide specific information regarding selective growth cone guidance leading to correct targeting.

Biochemical and immunocytochemical mapping studies identified a 64–68 kDa glycoprotein, the limbic system–associated membrane protein (LAMP), that is expressed by cortical and subcortical neurons comprising the limbic system (Levitt, 1984; Zacco et al., 1990). These brain areas form functional circuits involved in memory, learning, cognitive behavior, and central autonomic regulation. LAMP immunoreactivity is present early during development on neurons and transiently on growth cones and axons during pathway formation and differentiation (Horton and Levitt, 1988; Keller and Levitt, 1989; Zacco et al., 1990). From the unique patterns of LAMP immunoreactivity, we suggested that LAMP may serve as a critical component of recognition during circuit formation in the mammalian brain.

Considering the potentially important role that LAMP may play in vertebrate brain development, we undertook the present study to characterize its molecular and functional properties. We report the isolation of cDNA clones with the complete coding region of LAMP and characterize the protein as a new member of the IgSF with a high degree of homology to two other proteins containing a glycosyl–phosphatidylinositol (GPI) anchor and three-domain Ig structure. LAMP exhibits some features typical of cell adhesion molecules (CAMs), but is thus far unique among vertebrate CAMs in its ability to selectively induce growth of limbic neurons. This specificity of function is likely to be required for the normal development of an intrahippocampal pathway, as is evident by in vitro studies demonstrating selective adhesive properties and in vivo perturbation experiments administering LAMP antibodies postnatally.

Results

LAMP Is a New Member of the IgSF

Two separate preparations of affinity-purified LAMP yielded N-terminal sequences of 18 and 21 amino acids (Figure 1C, underlined), which were used to design degenerate primers. Four cDNA clones encoding LAMP were isolated by screening a cDNA library from adult rat hippocampus. These were subsequently amplified, subcloned, and sequenced, showing identical inserts of 1238 bp. The sequence of the *lamp* cDNA contains an open reading frame of 1014 bp that starts at the first methionine codon within a strong consensus sequence for initiation of translation (Kozak, 1987).

The predicted protein sequence derived from the open reading frame produces a 338 amino acid polypeptide (Figures 1A and 1C) with a calculated molecular mass of

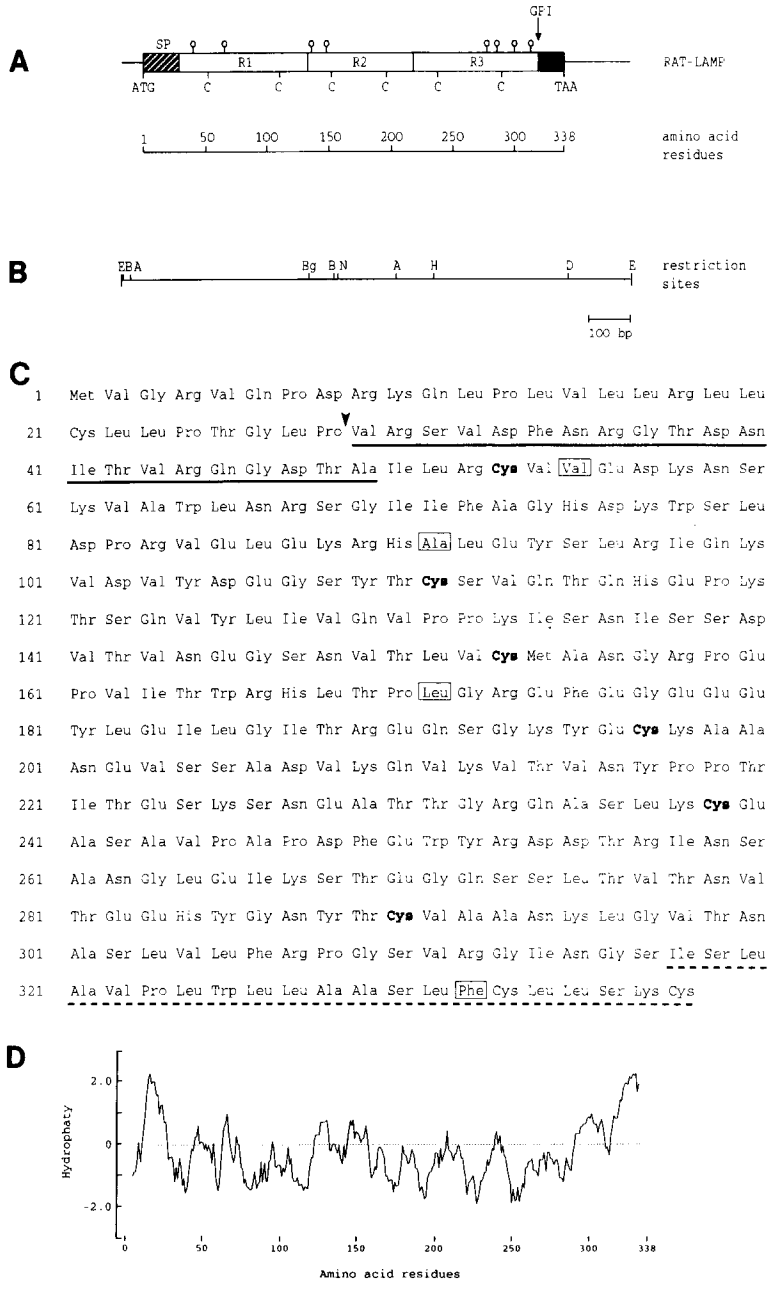

Figure 1. The Nucleotide Sequence of *lamp* cDNA Encodes a Polypeptide with Three Ig-like Domains and a GPI Anchor

(A) Schematic representation of *lamp* mRNA. The mRNA is represented in the 5′ to 3′ orientation with its protein coding region (1014 bp) shown as an open box. The translated open reading frame predicts a 338 amino acid polypeptide with a signal peptide (SP, hatched box) and a hydrophobic C-terminus (closed box) required for the processing of GPI-anchored molecules. LAMP contains three internal repeats (R1–R3), similar to the Ig domains, with conserved pairs of cysteine residues (C). There are eight putative N-glycosylation sites (open circles).

(B) Selected restriction sites are as follows: A, AccII; B, BalI; Bg, BglI; D, DraI; E, EcoRI; H, HindIII; N, NarI.

(C) Predicted amino acid sequence of rat *lamp* cDNA. The N-terminal amino acid sequence of the native protein, obtained by microsequencing, is underlined (thick line) with the predicted cleavage site for the signal peptide denoted by the arrowhead. The conserved cysteine residues of the Ig domains are shown in bold, and the hydrophobic C-terminal sequence is indicated by a dashed line. The amino acid substitution in the human sequence is indicated by the open box.

(D) Hydrophobicity analysis of LAMP predicted amino acid sequence indicates a hydrophilic polypeptide with hydrophobic domains representing the N-terminal signal peptide and the C-terminal sequence.

37 kDa. Hydrophobicity analysis (Kyte and Doolittle, 1982) indicates that the protein is mostly hydrophilic, with hydrophobic domains at both the amino and carboxyl ends (Figure 1D). The hydrophobic N-terminal sequence (amino acids 2–28) has the characteristics of a signal peptide with a predicted cleavage site after Pro28 (Figure 1C, arrowhead; von Heijne, 1986). Consistent with proteolytic processing of the signal peptide, Val29 would become the N-terminal amino acid of the mature protein followed by a predicted amino acid sequence identical with that determined for the purified native protein by microsequencing (Figure 1C). The C-terminus of LAMP contains a short stretch of hydrophobic amino acids (Figure 1C, dashed line), which is a common feature of proteins that are linked to the plasma membrane by a GPI anchor. Generally, this hydrophobic domain is removed by cleavage at a site of a small amino acid, which for LAMP is probably Asn315 (Ferguson and Williams, 1988; Cross, 1990; Gerber et al., 1992), with concomitant addition of the GPI moiety to the C-terminus. Further evidence that LAMP is GPI anchored is provided by the release of the recombinant protein expressed on the surface of transfected CHO cells by phosphatidylinositol-specific phospholipase C (PI-PLC) treatment (see Figure 4B). This agrees with previous biochemical evidence for linkage of native LAMP to the neuronal membrane via a GPI anchor (Zhukareva and Levitt, 1995).

The predicted molecular mass of LAMP after cleavage of the signal peptide and attachment of the GPI anchor

Table 1. Homology of LAMP with Immunoglobulin Superfamily Molecules

	Residues of Protein	Residues of LAMP	Percentage Identity	Opt. Score	z value
OBCAM	5–338	3–332	55.2	983	94.7
Neurotrimin	3–343	1–337	54.6	936	101.1
Amalgam	77–324	71–307	31.3	332	9.3
MAG	234–407	126–305	30.4	221	9.5
Lachesin	14–319	17–306	29.1	342	11.0
N-CAM	260–499	86–310	27.0	282	20.0
SMP	229–404	122–303	25.7	193	12.3
Tag-1	274–505	77–305	25.5	234	9.4
Fas II	1–317	2–305	23.9	247	9.3
L1	247–500	37–294	21.3	180	11.6
Ng-CAM	405–670	16–289	21.3	140	11.9

A search of the Swissprot database with the amino acid sequence of LAMP using the program FASTA (Williams, 1987) revealed significant similarity to cell adhesion molecules of the IgSF. The percentage of amino acid identity between LAMP and these proteins in their most homologous regions are shown. The greatest degree of similarity was found between LAMP and bovine and rat OBCAM and neurotrimin amino acid sequences. The significance of the homology is indicated by the Z value; Z values >6 are probably significant, and Z >10 are definitively significant.

is 32 kDa, considerably lower than the apparent molecular mass of 64–68 kDa determined for the native glycoprotein by SDS-PAGE (Zacco et al., 1990). The recombinant protein produced in E. coli has an apparent molecular mass similar to the predicted core protein (data not shown). The presence of eight putative N-linked glycosylation sites (Asn-X-Ser/Thr) (Figure 1A) suggests that extensive glycosylation of the protein backbone may account for the size discrepancy. Indeed, the recombinant protein, when produced in eukaryotic cells (CHO), has an apparent molecular mass similar to that of the native protein purified from the hippocampus (see Figure 4B). In erythropoietin, for example, the presence of oligosaccharides account for 50% of the protein molecular weight (Takeuchi and Kobata, 1991). The LAMP core protein also contains 11 potential phosphorylation sites (data not shown).

The deduced amino acid sequence contains three internal repeats of 99, 85, and 92 amino acids, 18%–26% homologous to each other in pairwise comparisons, with the highest identity centered around pairs of conserved cysteine residues that resemble those found in the Ig domains of the immunoglobulins and members of the IgSF (Williams, 1987; Williams and Barclay, 1988). Amino acid sequence comparisons using the FASTA program (Pearson and Lipman, 1988) revealed the most significant homologies to be with IgSF CAMs (Table 1). Moderate sequence identity (21%–31%), common among members of the IgSF, was found between LAMP and Amalgam (Seeger et al., 1988), myelin-associated glycoprotein (Arquint et al., 1987; Lai et al., 1987; Salzer et al., 1987), Lachesin (Karlstron et al., 1993), neural CAM (Cunningham et al., 1987), Schwann cell myelin protein (Dulac et al., 1992), TAG-1 (Furley et al., 1990), Fasciclin II (Grenningloh et al., 1991), L1 (Moos et al., 1988), and Ng-CAM (Burgoon et al., 1991). In contrast, we identified unusually high sequence identity (approximately 55%) to two IgSF members, which also have three Ig-like domains and a GPI anchor, the bovine and rat opioid-binding CAM (OBCAM; Lippman et al., 1992; Schofield et al., 1989), and neurotrimin (Struyk et al., 1995). Sequence analysis of the three Ig domains of LAMP in relation to other representative members of the IgSF allowed the assignment of these domains to the C2 set, even though the first domain showed some similarity with the variable region consensus (Williams, 1987; Williams and Barclay, 1988). Detailed analysis of the homology of LAMP with OBCAM and neurotrimin showed that domains one and two share 60%–62% identity, and the third Ig domain and the N-terminus share 47%–49% identity. Based on the high homology, we propose that these three molecules represent a new subclass in the IgSF that we designate as IgLONs (LAMP, OBCAM, neurotrimin subfamily).

Conservation of individual IgSF molecules among mammalian species is common. This characteristic, in addition to the distribution of LAMP immunoreactivity in highly conserved limbic regions of the brain, suggests that the LAMP molecule should retain critical structural features in the human. The human homolog of LAMP was cloned by RT-PCR using human cerebral cortex RNA in combination with oligonucleotide primers derived from the rat *lamp* sequence to produce several overlapping PCR products that were subcloned and sequenced. Sequence analysis reveals greater than 99% identity, with only four amino acid substitutions (Figure 1C, boxed residues), one of them located in the cleaved hydrophobic C-terminus. The analysis underscores the high conservation between human and rodent LAMP. Finally, genomic Southern blot analysis shows that in human and rat, a single copy gene encodes LAMP (data not shown).

lamp Exhibits Unique Expression among Limbic-Associated Structures

Northern blot analysis, using an antisense riboprobe, revealed two major transcripts of 1.6 kb and 8.0 kb in adult rat hippocampus, perirhinal cortex, and cerebellum, but none in nonneural tissues (Figures 2A and 2B, lane 1). These two bands were also detected with a 53-mer oligonucleotide probe complementary to a region that has relatively lower homology with other CAMs compared with the riboprobe (Figure 2B, lane 2). The deduced amino acid sequence of LAMP indicates that both the 1.6 and 8.0 kb transcripts could readily encode this protein, and that the

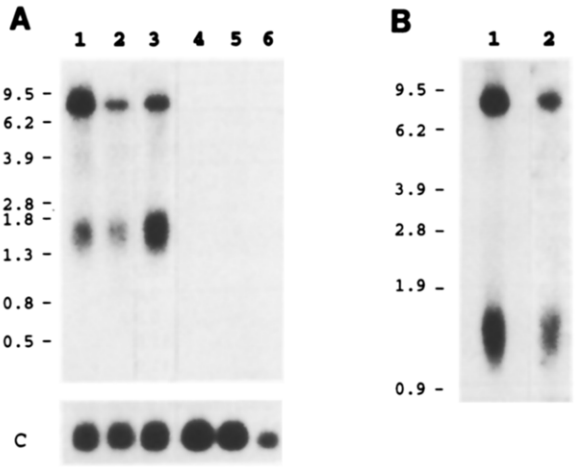

Figure 2. Northern Blot Analysis of *lamp* Gene Expression

(A) Distribution of *lamp* transcripts in adult rat indicates the presence of two transcripts of 1.6 and 8.0 kb in neural tissue (hippocampus [lane 1], perirhinal cortex [lane 2], and cerebellum [lane 3]), but not in nonneural peripheral tissue (kidney [lane 4], lung [lane 5], and liver [lane 6]).

(B) High resolution blots of hippocampal mRNA were hybridized with the antisense riboprobe (lane 1) and with an oligonucleotide (lane 2) derived from a region with minimal homology to OBCAM. Both probes recognized the 1.6 and 8.0 kb transcripts. Cyclophilin probe (c) was used as control for mRNA integrity and amounts of mRNA loaded.

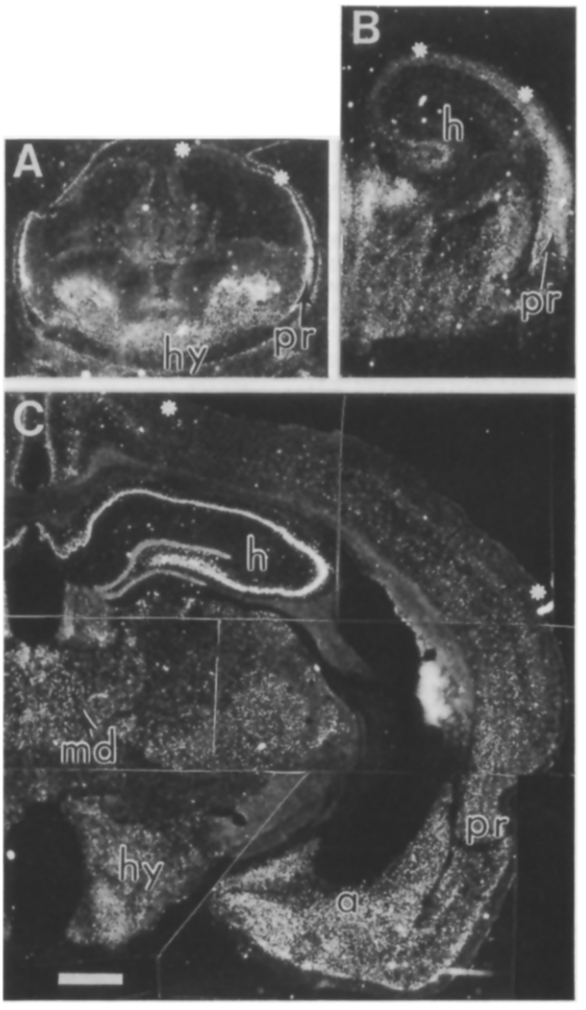

Figure 3. Darkfield Photomicrographs Illustrate the Specific Distribution of *lamp* Transcripts in the Developing and Adult Rat Brain

(A) Coronal section through the forebrain at E16 shows intense hybridization in the limbic perirhinal cortical region (pr) and hypothalamus (hy) and low signal in dorsal sensorimotor cortex (between asterisks).

(B) At the end of gestation (E20), the expression of the *lamp* transcript is high in the perirhinal region of cortex (pr) and sparse in the dorsal, nonlimbic cortex (between asterisks). The developing hippocampus (h) and midthalamic region, including the mediodorsal nucleus of thalamus (md), also exhibit intense hybridization.

(C) The specific expression of *lamp* transcripts remains in the adult brain, with intense hybridization signal in perirhinal cortex (pr), amygdala (a), hypothalamus (h), and medial thalamic region (md). Sparse signal is detected in sensorimotor cortex (between asterisks). Hybridization performed with riboprobes that spanned areas with high homology to *obcam* and *neurotrimin* resulted in patterns that overlapped with LAMP immunostaining, but also included areas lacking LAMP, such as olfactory bulb (data not shown).

Bar, 1 mm.

difference between these transcripts resides in the untranslated region. The same bands were also detected with human brain mRNA blots (data not shown).

In situ hybridization using the oligonucleotide probe showed very close, though not identical, correspondence between distribution of the *lamp* transcript and protein immunoreactivity. The general patterns of hybridization indicate relatively high expression in classic limbic regions and, with some exceptions, lower or no detectable expression in primary sensory and motor regions. Structures receiving mixed inputs, such as the striatum, exhibit patterns of hybridization that reflect LAMP immunostaining and known patterns of limbic input (Chesselet et al., 1991). During development, *lamp* hybridization is seen as early as E15–16 in the rat forebrain (Figure 3A), with dense expression in presumptive limbic cortex, basal forebrain, and hypothalamus. There is little hybridization in more dorsal, nonlimbic cortex. Later in gestation, the expression in presumptive limbic cortex remains very high, and medial limbic areas of the thalamus and hypothalamus are also strongly hybridized (Figure 3B). In the adult, the specific distribution of *lamp* transcripts in the cerebral cortex and subcortical limbic areas is similar to patterns seen prenatally (Figure 3C), with very dense expression in perirhinal, hippocampal, cingulate, amygdala, and limbic thalamic neurons and little to no expression in primary sensory areas of the neocortex. In contrast to the immunostaining (Levitt, 1984; Levitt et al., 1986), we found more expression of the *lamp* transcript in sensory thalamic nuclei, including the medial and lateral geniculate, although close examination reveals relatively fewer positive neurons (and less densely hybridized) in these regions than the heavily labeled medial limbic regions or neighboring hypothalamus.

LAMP Is a Selective Adhesion Molecule

To examine the functional properties of LAMP, we produced the recombinant LAMP in a mammalian system that is capable of faithful synthesis of the protein. Isolation of

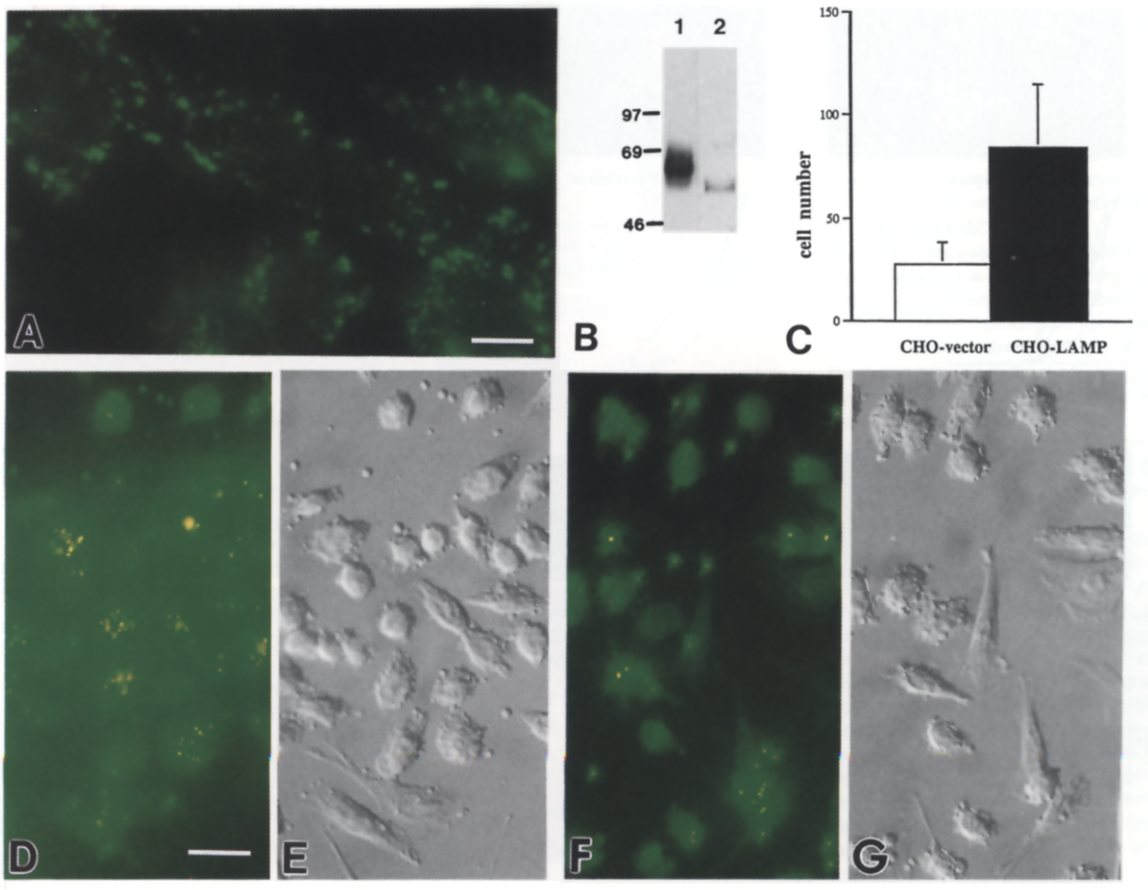

Figure 4. Recombinant LAMP Exhibits Selective Adhesive Properties

(A) Live CHO cells stably transfected with *lamp* construct (CHO_L) are immunoreactive with the antibody that recognizes the native form.

(B) Lane 1, native LAMP, released by PI-PLC treatment of hippocampal membranes, runs as a broad band between 64–68 kDa. Lane 2, the same treatment of the CHO_L cells releases all LAMP and runs as a more discrete band of approximately 55 kDa.

(C) Quantitative analysis of the specific binding of LAMP-coated Covaspheres to CHO_L-transfected cells compared with control, CHO vector-transfected cells (CHO_V). The number of cells in five equal fields per coverslip that had six or more beads bound were counted, and the mean of six coverslips in each category is displayed.

(D) Fluorescence micrograph depicts specific binding of LAMP-coated beads to the underlying CHO_L cells.

(E) Brightfield image of cells in (D).

(F) Fluorescence micrograph shows few LAMP-coated Covaspheres binding to the CHO_V cells.

(G) Brightfield image of cells in (F).

Bars, 5 μm (A); 20 μm (D–F).

stable transfectants yielded lines of CHO cells that express high levels of GPI-anchored LAMP, characterized by punctate immunoreactivity (Figure 4A) that is identical to the expression pattern of LAMP on the surface of cultured neurons. Treatment of the cells with PI-PLC results in release of the protein into the supernatant. The recombinant LAMP has an apparent molecular mass of 55 kDa (Figure 4B, lane 2), which is smaller than its native counterpart but larger than the polypeptide backbone. The recombinant protein in CHO cells thus appears to be partially postranslationally modified consistent with reports that some glycosyltransferases are silent in host cells (Warren, 1993). Nonetheless, recombinant LAMP exhibits functional activity (see below). Native, immunoaffinity-purified LAMP exhibits homophilic binding (Zhukareva and Levitt, 1995). Indeed, we found that Covaspheres coated with native LAMP also can bind to its recombinant counterpart on the transfected CHO cells (Figures 4C–4G). This homophilic property suggests that LAMP may have the ability specifically to regulate growth of limbic neurons. To explore this hypothesis, embryonic neurons from LAMP-expressing hippocampus and perirhinal cortex and non-LAMP-expressing olfactory bulb and visual cortex were plated on a substratum of LAMP-transfected (CHO_L) or vector-transfected (CHO_V) CHO cells. Encountering recombinant LAMP, both limbic populations showed extensive neurite outgrowth within 24 hr, exhibiting well-differentiated morphologies and often extending long neurites (Figure 5). These same neuron populations always grew poorly on the CHO_V cells. Quantitative analysis of process lengths showed that the neurites on the CHO_L substratum are longer and with a substantial subpopula-

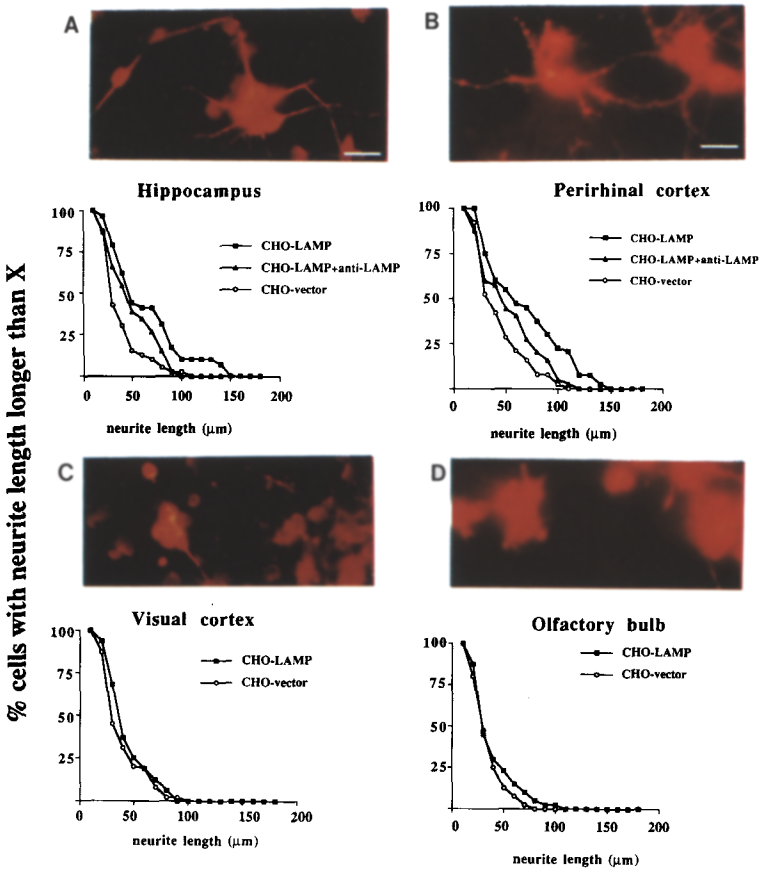

Figure 5. Analysis of Neurite Outgrowth of Limbic and Nonlimbic Primary Neurons on *lamp*-Transfected (CHO_L) and Vector-Transfected (CHO_V) Cells

(A) Hippocampal neurons grown on CHO_L substrate have longer neurites compared with growth on the CHO_V substrate. Anti-LAMP treatment of the cells plated on CHO_L eliminates the population of longest neurites (>100 μm) and reduces the number between 50 and 100 μm. Fluorescence photomicrograph above depicts well-differentiated MAP2-immunostained hippocampal neurons with long neurites on CHO_L substrate.

(B) Perirhinal limbic cortical neurons exhibit same specific outgrowth on CHO_L substrate as hippocampal neurons. In the photomicrograph, perirhinal neurons prelabeled with dye show similar neurite outgrowth as seen with MAP2 staining.

(C) Nonlimbic cells from occipital (presumptive visual) cortex exhibit the same pattern of neurite outgrowth on the two CHO substrates, with a noted absence of a neurite population greater than 100 μm on either substrate. Photomicrograph of MAP2-stained neurons on the CHO_L substrate reveals very short neurites. Magnification is same as in (A).

(D) Nonlimbic olfactory cells have similar neurite outgrowth profiles on CHO_L and CHO_V substrates. Note the absence of a neurite population greater than 100 μm. Photomicrograph depicts a group of dye-labeled cells with a few, very short neurites. Magnification is same as in (B).

Bars, 20 μm (A); 15 μm (B).

tion exceeding 100 μm (Figure 5). We further documented the specificity of the LAMP interaction by preincubating limbic neurons with functionally blocking LAMP antibodies prior to plating. This treatment resulted in a significant reduction of neurite length on the CHO_L cells (Figure 5). The effect was particularly evident for the population of longer neurites, which did not form following antibody exposure. The selective nature of LAMP-mediated outgrowth was illustrated by analysis of the behavior of nonlimbic cell populations. Both olfactory and visual neurons bound to the CHO_L substratum, but in contrast to their limbic counterparts, differentiated poorly and extended shorter neurites, which usually did not exceed 80 μm (Figure 5). In addition, neurites of the nonlimbic neurons grew almost identically on CHO_L and the control CHO_V cells, reflecting the absence of a specific, growth-enhancing interaction between the LAMP-expressing cell line and nonlimbic neurons.

LAMP Controls Axon Patterning in an Intrahippocampal Circuit

To study the effects of blocking LAMP function on a developing limbic circuit, we focused on a late-developing pathway in the hippocampus. Previous antibody perturbation in vitro showed that anti-LAMP can prevent axon targeting in the septo-hippocampal pathway (Keller et al., 1989). This circuit, however, develops prenatally, making it difficult to perturb in vivo with antibodies. In contrast, the excitatory glutaminergic mossy fiber projection of granule cells to pyramidal neurons begins to develop at birth and continues postnatally over a 3 week period in rats (Bliss et al., 1974; Amaral and Dent, 1981). Three features make this pathway amenable to manipulation with antibodies. First, we have shown that growing mossy fiber axons, growth cones, and their target pyramidal neuron dendrites express LAMP during development (Keller et al., 1989; Zacco et al., 1990). LAMP is maintained only on postsynaptic perikarya after synapse formation. Second, the ingrowth by mossy fibers occurs subsequent to the early, orderly innervation of pyramidal cell dendrites by commissural and entorhinal afferents. Third, the developing mossy fibers, which can be visualized most readily and specifically with the Timm histochemical stain, navigate in a stereotypic manner along a well-defined pathway that leads to the innervation of a discrete zone in the regio inferior of the hippocampus, the proximal portions of the apical dendrites of the pyramidal neurons (Bliss et al., 1974; Gaarskajaer, 1985; Ribak and Navetta, 1994). We injected animals intraventricularly four times during the first postnatal week either with control IgG, an anti-L1 monoclonal antibody (Sweadner, 1983) that binds to the membrane of developing axons, or anti-LAMP. Most animals were analyzed at the approximate midpoint of development of the mossy fiber pathway, postnatal day (P) 9–12. When exposed to either nonspecific IgG or anti-L1, the mossy fibers projected normally, with little or no growth through the stratum pyramidale or stratum oriens (Figures

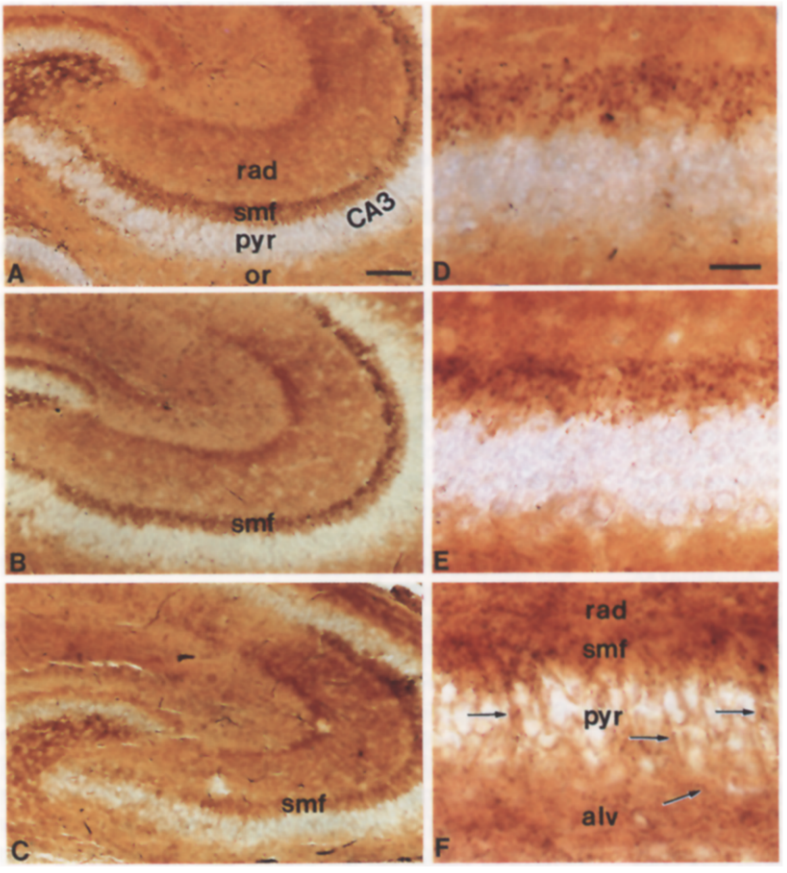

Figure 6. Direct Treatment of Postnatal Rats with Anti-LAMP Administered Intraventricularly on Alternating Days Results in Aberrant Growth of the Mossy Fiber Projection in the Hippocampus

Fibers visualized with the Timm stain in horizontal sections through the hippocampus. Low power photomicrographs of hippocampus from P9 rats injected with control IgG (A), anti-L1 (B), and anti-LAMP (C).

(A and B) The control and L1 antibody treatments result in a normal pattern of darkly stained suprapyramidal mossy fibers (smf), located in a tightly fasciculated zone within the stratum lucidum of CA3. Note the absence of fibers and boutons in the pyramidal cell zone, beneath in the stratum oriens (or), or above in the stratum radiatum (rad).

(C) At this low magnification, the Timm-stained pattern appears more diffuse than normal, with histochemical product in both supra-and infrapyramidal regions.

(D and E) Higher magnification photomicrographs of CA3 pyramidal zone from (D) IgG control and (E) anti-L1 animals. Both exhibit a normal mossy fiber projection, which is tightly arranged along the proximal suprapyramidal zone.

(F) Photomicrograph through the hippocampus from a P9 animal injected with anti-LAMP. There is aberrant growth of Timm-stained fibers throughout the CA3 region. Note the boutons and extensive fiber network (arrows) in regions that normally never contain mossy fibers, including stratum pyramidale (pyr), stratum radiatum (rad), and the alveus (alv).

Bar, 100 μm (A–C); 30 μm (D–F).

6A, 6B, 6D, and 6E; Figure 7A). In rats treated with anti-LAMP, however, we observed marked changes in the developing mossy fiber pathway. At low magnification, the developing projection appears uncharacteristically diffuse (Figures 6C and 6F; Figure 7B). Detailed examination at higher magnification, at which individual processes and varicosities can be seen, shows that misdirected axons traverse through the stratum pyramidale and oriens, as well as across the entire plexus zone of the stratum radiatum. In many animals, misrouted axons even entered the alveus, a pathway that normally carries fornix and commissural axons. Quantitative analysis comparing the normal and perturbed growth was performed in stratum pyramidale. In control animals, the calculated area fraction value is 0.05 ± 0.002, indicating that 5% of the area analyzed is occupied by Timm-stained profiles. Anti-LAMP treatment results in an area fraction value of 0.29 ± 0.006, a 6-fold increase in the area occupied by the aberrant mossy fiber projections ($p \leq .001$; t test). Limbic pathways that form prenatally, such as the septo-hippocampal cholinergic projection, are normal in the animals treated with anti-LAMP. The LAMP perturbation in vivo thus produces a significant departure from the normal patterning of the hippocampal mossy fiber projection.

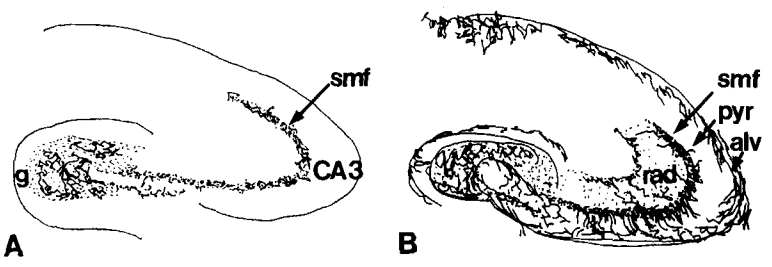

Figure 7. Camera Lucida Drawing of the Distribution of Mossy Fibers in the Normal or Anti-LAMP-Exposed Hippocampus at P9 from Two Animals

(A) Normal hippocampus; (B) anti-LAMP-exposed hippocampus.

The abnormal growth depicted here represents the more extreme phenotype following 1 week of anti-LAMP exposure. The restricted route of mossy fiber growth from the dentate gyrus into Ammon's Horn in the normal hippocampus is disrupted after anti-LAMP treatment, with fibers entering stratum radiatum (rad), pyramidal (pyr), and infrapyradimal zones, extending into the alveus (alv).

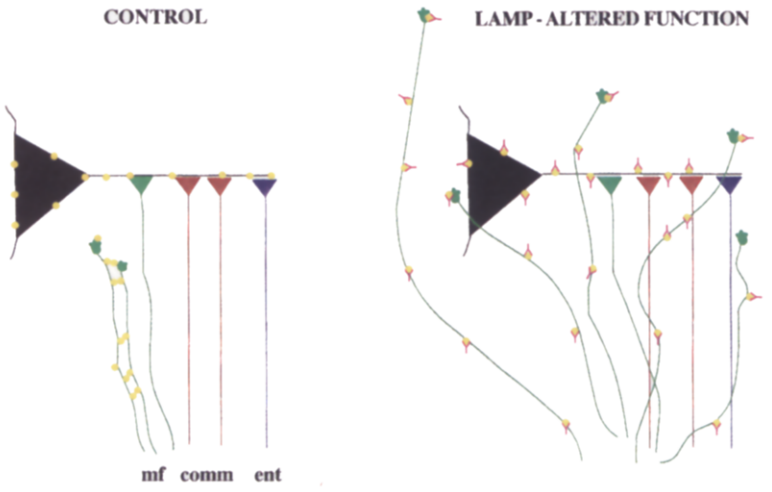

Figure 8. Summary Drawing Depicts Hypothesized Role for LAMP in Mossy Fiber Development

Mossy fibers (mf) normally fasciculate via homophilic LAMP interactions (yellow dots) in a restricted band of growth in the suprapyramidal zone. The earlier arriving commissural (comm) and entorhinal (ent) axons do not express LAMP upon synaptogenesis. In the LAMP-altered phenotype, the antibody (pink) causes mossy fiber defasciculation, resulting in atypical growth patterns into other zones and pathways. Note the absence of modification of the afferents that had arrived prior to the extended period of antibody exposure.

Discussion

Axon growth that occurs along defined pathways, and their subsequent invasion into specific targets to form functional circuits, has been proposed to be directed by unique molecular signals (Goodman and Shatz, 1993). In the invertebrate nervous system, distinct temporal and spatial expression of membrane-associated proteins is an important characteristic underlying the specificity of connections that form during development. Perturbation of the genes encoding these proteins has led to mutations in defined neural circuits (Goodman, 1994; Lin and Goodman, 1994). We have described the cloning of a new member of the IgSF, LAMP, and we have shown, using different experimental paradigms, that LAMP serves as an important mediator of axon patterning in the developing limbic system in the vertebrate brain.

Most CAMs exhibit a high degree of homology among forms of the proteins, indicative of conservation of function. LAMP is 99% homologous between rat and human, with transcripts of identical size encoding the protein, suggesting that all structural elements required for unique molecular recognition are retained. The homology between LAMP and most other CAMs of the IgSF is relatively moderate, probably reflecting structural features of the conserved sequences surrounding the cysteine pairs of the Ig domains required to form the β-pleated structure. In contrast, we found a strong relationship between LAMP and two other IgSF members that also have a three-domain loop structure, OBCAM and neurotrimin. All three are GPI anchored and exhibit very high homology within the first two domains. It is in the third loop that diversity among these subfamily members is expressed, and thus it is likely to represent the region that defines the functional specificity of each. Restriction analysis of the *lamp* gene reveals differences when compared with *neurotrimin*. This information is currently not available for *obcam*. In addition, the size of the transcripts encoding *lamp* are distinct from those identified with *neurotrimin* and *obcam* probes. Given the similarities between domains 1 and 2, it is likely that the three members of this subfamily arose through duplications that also resulted in specific structural divergence, providing diversity in expression patterns and function.

In a controlled environment, such as on CHO_L-transfected cells or a pure LAMP substrate (Zhukareva and Levitt, 1995), we showed that LAMP enhances outgrowth, but only for limbic neurons. The ability of antibodies to block this cellular response suggests that LAMP promotes homophilic binding. CAMs, however, can act through both homophilic and heterophilic mechanisms (Felsenfeld et al., 1994). Though heterophilic interactions between LAMP and another surface molecule also could contribute to the specificity that we report here using the in vitro assay, the relatively modest outgrowth of nonlimbic neurons on a LAMP substrate would require that a second molecule be expressed uniquely by limbic neurons. Under more complex conditions, such as in vivo or in explants, antibody perturbation does not disrupt cell differentiation or general fiber outgrowth, indicating that in the presence of other tropic molecules and CAMs, LAMP is not required for initiation or maintenance of axon growth. Rather, through homophilic interactions, LAMP probably participates in neuronal recognition that contributes to patterning of connections.

Though only correlative, the distinct anatomical patterns of LAMP expression during fetal development, shown immuncytochemically (Horton and Levitt, 1988; Ferri and Levitt, 1993) and here by in situ hybridization, are consistent with LAMP playing a role in the molecular recognition between groups of functionally related neurons. We initially tested this model using two different experimental paradigms, fetal tissue transplants and in vitro antibody perturbation. Using transplants of fetal cells, we were able to induce LAMP expression by grafted neurons that normally do not synthesize the protein (Barbe and Levitt, 1991). The resulting thalamo-cortical and cortico-cortical connections formed by the transplanted neurons with the host brain specifically reflect their new limbic phenotype (Barbe and Levitt, 1992, 1995). In explants, we showed that antibodies against LAMP prevented the invasion of the hippocampus by normal afferents, the septal cholinergic axons, resulting in a misrouted projection that grew

past their target (Keller et al., 1989). In the present study, we created a misrouted mossy fiber projection in the hippocampus in vivo using anti-LAMP, but failed to modify the developing projection with an anti-L1 monoclonal antibody. Similar to the results using explants, we did not find that anti-LAMP caused a reduction in fiber outgrowth or a major defect in the general direction of outgrowth. Rather, the mossy fibers grew in a less restricted fashion, extending through hippocampal zones that they normally never enter (Figure 8). Clearly, the mossy fibers use cues other than LAMP to extend from the dentate gyrus, but in the presence of LAMP antibodies, the fibers do not maintain the precision normally seen in this well-organized route. Imprecision in the development of the mossy fiber projection is reminiscent of mutations produced recently in Drosophila (Lin and Goodman, 1994). Loss-of-function *fasII* mutations result in decreased fasciculation of axons, but *fasII* does not play a major role in controlling the general direction and extent of neurite outgrowth. In some animals exposed to anti-LAMP during the first week and then examined as adults, we found similarly that the altered mossy fiber projection recovered. The loss-of-function *fasII* and LAMP phenotypes also are comparable to that seen with neurotransmitter-induced down-regulation of axonal apCAM in Aplysia, which led to a decrease in axon fasciculation and an increase in axon branching (Peter et al., 1994). Given the distribution of LAMP on all developing pyramidal neurons and dendrites in the hippocampus, the selective guidance role of the protein in normal mossy fiber development may involve the facilitation of axon–axon interactions among LAMP-positive processes when granule cell neurites first form postnatally. The restricted growth of mossy fibers into the proximal dendritic field probably reflects a combination of the selective adhesion among late-developing, LAMP-expressing mossy fibers and the occupation of the more distal dendritic fields by earlier forming entorhinal and commissural inputs (Figure 8).

Models of molecular recognition have been postulated by Sperry (1963) and others (Goodman et al., 1984; Jessell, 1988). Together with CAMs that are found ubiquitously in the vertebrate brain, developmental specificity is likely to be achieved through the early, selective expression of different classes of molecules. In this classic model, restricted synthesis of repulsive and attractive tropic molecules, such as collapsin, netrins, and CAMs (Kennedy et al., 1994; Serafini et al., 1994; Tessier-Lavigne, 1994), participate in pathway choices that subsequently lead to the involvement of selectively expressed adhesive proteins, such as LAMP, in target recognition. Defects in the tropic class would likely result in misguided axons that never reach their target, as seen in the netrin homolog *unc-6* mutation in C. elegans (Hedgecock et al., 1990; Ishii et al., 1992). We suggest that the absence of a putative targeting molecule like LAMP would produce somewhat more subtle defects, in which basic pathways form normally but contain growing fibers that bypass or grow through or around their normal fields of termination. In this context, the disrupted LAMP phenotype reported here exhibits features similar to the reported abberant mossy fiber outgrowth following induced epileptic activity in the adult hippocampus (Houser et al., 1990). It is possible, therefore, that regulation of LAMP expression by physiological activity, as shown for apCAM, will affect adult limbic circuits. The cloning of LAMP provides us with an opportunity, through molecular genetic strategies, to define further the contribution of this and related IgLONs to the guidance of developing axons and remodeling of mature circuits in the limbic system.

Experimental Procedures

Cloning and Sequencing

LAMP was affinity purified from adult hippocampal membranes (Zacco et al., 1990), run on SDS-PAGE, and electroblotted onto PVDF membranes in preparation for peptide microsequencing (Matsudaira, 1987). Bands were sequenced on an Applied Biosystem 470A gas phase sequencer equipped with a 120A on-line PTH analyzer (Henzel et al., 1987). An adult rat hippocampus cDNA library constructed in the λgt11 expression vector (Clontech) was screened with ^{32}P end-labeled, degenerate oligonucleotide probes derived from two distinct regions of the N-terminal amino acid sequence of LAMP, VRSVDFNRGTDNITVRQGDTA. Group 1 was GAYTTYAAYCGIGGIACIGAY and GAYTTYAAYAGRGGIACIGAY; group 2, ATHACIGTICGICARGGIGAY and ATHACIGTIAGRCARGGIGAY; R = A/G, Y = C/T, H-A/C/T I = Inosine. Plaque purified clones that rendered positive for both probes had their cDNA inserts amplified by PCR (30 cycles: 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min) using forward and reverse lambda primers (Promega) and were subcloned into pCR II vector (Invitrogen). Human cerebral cortex cDNA (1 ng; Clontech) was amplified by PCR using a combination of primers complementary to the rat *lamp* nucleotide sequence. PCR products were subcloned into pCR II vector (Invitrogen) or pGEM-T vector (Promega). The nucleotide sequence was determined, on both strands, by the dideoxy chain termination method (Sanger et al., 1977). Unless otherwise indicated, all standard procedures were performed essentially as described by Sambrook et al. (1989) and Hockfield et al. (1994).

Northern Blot Analysis

Total cellular RNA was isolated from adult, Sprague-Dawley rats, and the poly(A)$^+$ RNA fraction was purified using the PolyATract mRNA isolation system (Promega). Poly(A)$^+$ RNA (3 μg) was separated on 1.5% (see Figure 2A) or 1.2% (see Figure 2B) agarose–formaldehyde gel, transferred to a nylon membrane (Nytran, Schleicher and Schuel), UV cross-linked, and hybridized overnight under stringent conditions with ^{32}P-labeled RNA probes. Antisense probes were transcribed in vitro using T7 RNA polymerase from *lamp* cDNA template linearized with BalI (nucleotide 464–1238), while control sense probes were prepared with SP6 RNA polymerase from a template digested with NarI (nucleotide −55–471). The oligonucleotide probe (nucleotide 918–970) was labeled by the addition of a ^{32}P poly(A) tail.

In Situ Hybridization

Tissue sections fixed with 4% paraformaldehyde were hybridized with the 53-mer oligonucleotide probe labeled by the addition of ^{35}S poly(A) tail. Hybridization was performed at 58°C, with probe concentration at 10^6 cpm/ml of hybridization buffer (50% formamide, 10% dextran sulfate, 0.2 M NaCl, 1 × Denhardt's solution, 10 mM Tris, 1 mM EDTA). High stringency posthybridization washes included 1 hr in 1 × SSC at 60°C. No signal was detected on sections hybridized with the sense oligonucleotide probe.

Cell Transfections and Bead Binding Assay

The rat *lamp* cDNA was subcloned from pCR II into the EcoRI site of the eukaryotic expression vector pcDNA3 (Invitrogen). CHO cells were transfected with 10–15 μg of pcDNA3–*lamp* using calcium phosphate precipitation. Transfectant clones were selected with 400 μg/ml active G418, and positive colonies were subcloned by limiting dilution. CHO cells transfected with pcDNA3 were used as negative controls. For localization of recombinant LAMP, live cells were incubated with mouse anti-LAMP, washed four times with DMEM/10% FCS, incubated with FITC-conjugated donkey anti-mouse following fixation with

4% formaldehyde, and mounted in glycerol/PBS with 5% propyl gallate. Fluorescent Covasphere beads (Duke Scientific) were incubated for 1 hr with PI-PLC-released, affinity-purified LAMP from hippocampal membranes. The coated beads were sonicated for 5 min immediately prior to plating on monolayers of transfected cells. Incubation continued for 30 min, followed by brief washes in culture medium, and then fixation in formalin solution. Counts of cells with more than six beads bound were made on each coverslip by examining five fields spaced 2 mm apart.

Neuronal Cultures on Tranfected Cells

Primary neurons from E16 embryos were prepared as described previously (Ferri and Levitt, 1993; Zhukareva and Levitt, 1995). Cells were resuspended in DMEM, and in some experiments, were labeled with lipophilic dye PKH26 (Sigma). Cells were plated in DMEM/10% FCS at the density 5×10^3 cells/ml on monolayers of CHO-transfected cells. After 48 hr in culture, coverslips were fixed with 4% formaldehyde, mounted, and examined under a fluorescence microscope. In some experiments in which cell prelabeling was not done, cultures were fixed and stained with the neuronal marker anti-MAP2, as described previously (Ferri and Levitt, 1993; Zhukareva and Levitt, 1995). Quantitative analysis was performed on dye-labeled and MAP2-stained cultures. The length of the longest neurite of 10–15 process-bearing cells, selected randomly, was measured on each of six coverslips prepared for each category in three different experiments. A Bioquant Image Analysis system was used to digitize and measure the images. The observer was blind as to the neuron population and substrate. Each point on the graph represents the percentage of total cells with neurites longer than X.

Antibody Application and Timm Stain

Sprague-Dawley rats (n = 34) were used. Anti-LAMP (n = 15) and control IgG (n = 14) anti-paramyosin (both IgG2a isotype) were affinity purified from hybridoma supernatant on a Protein A column. Anti-L1 (n = 5) was purified using a Protein A column and the MAPSII buffer system (Biorad) from ascites fluid (a gift of Dr. Jane Dodd, Columbia University College of Physicians and Surgeons), produced using the ASCS4 hybridoma cell line (Sweadner, 1983). Fab' fragments of all antibodies were made by digestion on immobilized papain (Pierce). Pure fragments (10 μg) were injected into the cisterna magna using a 32-gauge needle on P0, P2, P4, and P6. Animals were sacrificed on P9 by transcardial perfusion with 4.9% sodium sulphide (Na_2S) in phosphate buffer (pH 7.4). Brains were postfixed in Carnoy's solution with 1.2% Na_2S for 24 hr. Paraffin sections from control, anti-L1, and anti-LAMP treated groups were prepared for mossy fiber staining using the Timm method (Haug, 1973). For quantitative analysis, sections from control, L1, and LAMP antibody treated brains (n = 4 for each group) were stained in parallel and different subfields analyzed for density of innervation using the Bioquant OS/2 image analysis system. Six sections per brain at regular intervals were measured, with three fields per section measured.

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GenBank Accession Number

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