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Lachesin: an immunoglobulin superfamily protein whose expression correlates with neurogenesis in grasshopper embryos

Rolf O. Karlstrom, Leigh P. Wilder and Michael J. Bastiani

Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA

SUMMARY

We describe the developmental expression in grasshopper (*Schistocerca americana*) and molecular characterization in grasshopper and fruit fly (*Drosophila melanogaster*) of Lachesin, a novel immunoglobulin superfamily protein. Lachesin is expressed on the surfaces of differentiating neuronal cells from the onset of neurogenesis in both the central and peripheral nervous systems. Lachesin expression begins in some cells of the neurogenic ectoderm immediately after engrailed expression begins in the posterior cells of each future segment. All neurogenic cells express Lachesin early, but only those cells that become neuroblasts continue to express Lachesin. Ectodermal cells in the neurogenic region that adopt non-neuronal fates lose Lachesin at the time that they diverge from a potentially neurogenic pathway. Neuroblasts, ganglion mother cells and neurons all express Lachesin early in their lives, but expression becomes restricted to a subset of neurons as development progresses. Sensory neurons express Lachesin as they delaminate from the body wall ectoderm. Lachesin is also present on growing axons of the CNS and PNS and becomes restricted to a subset of axons later in development. This expression is unique among known insect neurogenic genes and suggests a role for Lachesin in early neuronal differentiation and axon outgrowth.

Grasshopper Lachesin is a 38×10^3 M_r protein linked to cell membranes through a glycosyl phosphatidylinositol anchor. We have cloned the *Lachesin* gene from both grasshopper and fly. The proteins are highly conserved (70% identical) between the two species. Lachesin is similar to *Drosophila* amalgam, bovine OBCAM and the human poliovirus receptor, putting it into a subgroup of the immunoglobulin superfamily containing one V- and two C2-type immunoglobulin domains. Lachesin is also similar to several other vertebrate immunoglobulin superfamily proteins (TAG-1, F11, L1 and NgCAM) known to function in neurite outgrowth and other cell surface recognition events.

Key words: insect neurogenesis, neuronal differentiation, neuroblast, axon outgrowth, grasshopper, immunoglobulin superfamily

INTRODUCTION

The nervous system is the most complex system in higher animals. Perhaps because of this complexity, some of the first differentiation events seen during embryonic development occur in the future nervous system. Very early in development, a large population of cells in the embryonic ectoderm becomes neurogenic, then cells within this population become progressively more specialized. Within the neurogenic ectoderm, some cells become neuronal precursors (neuroblasts), while others diverge from a neuronal developmental pathway to form non-neuronal elements of the nervous system. Throughout the process of neuronal differentiation, interactions between cells play a central role in the determination of cellular identity. These interactions must be mediated by molecules expressed on cell surfaces at appropriate times in development. The identification and characterization of cell surface molecules mediating these intercellular communication events is thus crucial to understanding the process of neuronal differentiation.

Insects are a particularly powerful system for studying the molecular mechanisms of early neurogenesis. As in vertebrates, neurogenesis in grasshoppers and fruit flies begins with an early segregation event in the ectoderm. The central portion of the ventral ectoderm becomes morphologically distinct from lateral non-neurogenic ectoderm and exhibits properties distinct from lateral ectoderm. Transplantation experiments show that ectodermal cells that normally differentiate along non-neuronal developmental pathways are able to undergo neuronal differentiation when moved into this region (Technau and Campos-Ortega, 1988). These cells are either induced to begin neuronal differentiation in the neurogenic ectoderm, or they may be released from a signal that inhibits neuronal differentiation in the body wall ectoderm. Surface molecules that mediate the intercellular communication necessary for these interactions have not been identified.

Within the neurogenic region of insects, an early pattern of approximately 150 potentially neurogenic cells in each hemi-segment is refined into a stereotyped pattern of

approximately 30 neuroblasts, their surrounding support cells and other non-neuronal cells (Doe et al., 1985). Ablation experiments in grasshoppers (Doe and Goodman, 1985b) have shown that cell interactions are responsible in part for this divergence of fate. Neuroectodermal cells can replace an ablated neuroblast, showing that differentiating neuroblasts inhibit neighboring cells from adopting neuronal fates. Very few surface molecules have been identified that may mediate the cellular interactions between neuroblasts and neuroectodermal cells. In *Drosophila*, Notch and Delta are two genetically identified surface proteins that play a role in lateral inhibition of neuronal differentiation by neuroblasts (reviewed in Artavanis-Tsakonis et al., 1991). Both proteins belong to a family of proteins containing epidermal growth factor (EGF)-like repeats and are part of a signaling mechanism that either prevents neuronal differentiation or causes epidermal differentiation (Hoppe and Greenspan, 1990; Xu et al., 1990). Notch and Delta are also involved in apparently similar mechanisms that lead to differentiation events in the insect peripheral nervous system (PNS; Heitzler and Simpson, 1991).

We have taken an immunological approach to finding new molecules involved in insect neurogenesis. By visualizing the distribution of cell surface molecules using monoclonal antibodies (mAbs), it is possible to identify molecules with patterns of expression consistent with a role in specific developmental processes. In this paper, we describe the spatially and temporally restricted developmental expression of the cell surface protein recognized by the 1C10 monoclonal antibody (mAb). Based on its expression pattern, we propose that the 1C10 antigen may be involved in the process of cellular fate determination during neurogenesis. The antigen is present on cells that are segregating from their neighbors to follow neuronal developmental pathways and is lost on cells that become non-neuronal. Because of its potential role in determining cell fate, we have called the protein Lachesin, after one of the three Greek fates, Lachesis.

Lachesin is expressed in neurogenic cells early in development, before morphological changes associated with neuronal differentiation are apparent. Lachesin is thus a very early neuronal marker and may be involved in cell interactions at the beginning of neurogenesis. Only cells that remain on neuronal developmental pathways maintain Lachesin expression, suggesting that Lachesin expression plays a role in neuronal differentiation but not in differentiation of non-neuronal cells. Later in development, the Lachesin expression pattern is consistent with a role in axonal outgrowth. We have cloned the *Lachesin* gene from both grasshopper and fruit fly. The two insect Lachesin proteins show a high degree of sequence conservation. Lachesin is a novel member of the immunoglobulin (Ig) superfamily of proteins with sequence similarity to other neuronally expressed cell adhesion molecules. The restricted expression of Lachesin on cells following neuronal developmental pathways, combined with sequence similarity to other Ig superfamily molecules, suggests Lachesin plays a role in early cell recognition and/or adhesive events necessary for the formation of the insect nervous system.

MATERIALS AND METHODS

Generation and screening of mAbs

The 1C10 mAb was found in the same mAb fusion as the 2B2 mAb (Seaver et al., 1991) and the 7F7 mAb (Carpenter and Bastiani, 1991). Briefly, BALB/c mice were immunized with dissected nerve cord membranes from 40% grasshopper embryos. Antibody-producing cells were fused with myeloma cells as previously described (Kohler and Milstein, 1975). Screening was done by incubating fixed 40% embryos in individual hybridoma supernatants overnight at 4°C, washing for 30 minutes in PBS, incubating in blocking solution for 15 minutes, then incubating 2 hours at room temperature in FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) diluted 1:100 in blocking solution. Embryos were washed 1 to 2 hours with several changes in PBS, then mounted in glycerol for viewing under a Leitz compound microscope using a 25× objective and epifluorescent illumination. Ascites fluid was generated as previously described (Harlow and Lane, 1988).

Immunohistochemistry

Embryos were fixed in 2% paraformaldehyde in Millonig's buffer for 30 minutes, washed in PBS 3× 10 minutes, then blocked 15 minutes in PBS+5% Goat serum+0.2% Triton X-100 (blocking solution) for 15 minutes. The embryos were incubated in 1C10 mAb ascites fluid at a dilution of 1:1000. For live labelling, embryos were dissected into Paul's culture medium (50% Schneider's *Drosophila* medium (Gibco), 40% RPMI (Gibco), 1.5 mg/ml bovine insulin (Sigma), 0.001 mg/ml juvenile hormone (Sigma) 0.001 mg/ml β-ecdysone (Sigma) and 10,000 U/ml antimycotic/antibiotic (Sigma) (Raper et al., 1984)). Embryos were then pinned flat and incubated in the 1C10 ascites 2 hours at room temperature in Paul's. Following this primary incubation, the antibody was washed off 2× 15 minutes in medium. Embryos were fixed 30-60 minutes as above, washed, blocked and labelled with a peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Peroxidase-labelled embryos were incubated 15 minutes in 1 mg/ml DAB, reacted in 0.003% H_2O_2, cleared in glycerol and viewed using Nomarski optics.

Embryos were treated for electron microscopy as previously described (Seaver et al., 1991; Carpenter and Bastiani, 1991). Briefly, live labelled embryos were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde for 30 minutes, washed in Tris-buffered saline (TBS; 50 mM Tris pH 7.2, 350 mM NaCl) and reacted with DAB using β-D-glucose and glucose oxidase to generate H_2O_2. After postfixation in 1% paraformaldehyde, 1% glutaraldehyde, embryos were incubated in 1% OsO_4 in TBS for 1 hour, washed in TBS, stained with uranyl acetate, dehydrated in graded ethanol and embedded in plastic. Ultrathin sections were mounted on Formvar-coated slot grids and viewed using a Phillips 201 electron microscope.

Immunoprecipitation

Embryonic membrane proteins were labelled and precipitated as described previously (Seaver et al., 1991) using 300-500 40% embryos. ^{125}I-labelled membrane proteins were precipitated with the 3B11 and 1C10 mAbs in that order. Precipitated pellets were washed as described and run on a 7.5% polyacrylamide gel under reducing conditions. The gel was dried and placed on Kodak X-OMAT X-Ray film.

Western immunoblotting

Embryonic membranes were run on a 10% polyacrylamide gel and transferred to nitrocellulose membranes. The blotted protein was labelled with india ink and cut into strips, each containing one lane of protein. The strips were incubated overnight in blocking solution (universal buffer (UB=150 mM NaCl, 0.1% NaN_3, 50 mM Tris-

HCl pH 7.6) containing 2.5% BSA, 0.05% Tween 20 and 0.2% gelatin). The strips were then incubated 2 hours at room temperature in primary antibody diluted 1:5000 in blocking solution. Strips were washed 6× 5 minutes in wash solution (blocking solution without BSA), blocked 1 hour and incubated in rabbit anti-mouse secondary antibody diluted 1:1000 in blocking solution. After washing again 6× 5 minutes and blocking 30 minutes, the strips were incubated 30 minutes in ^{125}I-labelled protein A (Amersham). After a final series of washes, the strips were dried and placed on Kodak X-OMAT X-ray film.

PI-PLC treatment

PI-PLC treatment was performed essentially as in Chang et al. (1992). PI-PLC was a generous gift from Dr Martin Low, Columbia University. Phosphatidylcholine-specific PLC (PC-PLC) was from Boehringer Mannheim. Embryos were dissected into sterile Ringer's (+ 10 mM glucose) then cultured for 2 hours at 30°C in Paul's medium (without calf serum, insulin and hormones) in the presence or absence of 1.2 U PI-PLC/ml with agitation every 30 minutes. The media was removed and concentrated in centricon-10 microconcentrators. After removing some embryos for antibody labelling, embryonic membrane proteins were prepared (Seaver et al., 1991). Samples were run on a 12.5% polyacrylamide gel and blotted to nitrocellulose for western analysis.

Immunoaffinity purification

Lachesin was purified from grasshopper embryonic lysates by passage over a monoclonal affinity column. 1C10 mAb was coupled to 1 ml protein G-coated Sepharose 4B beads (Pharmacia) using dimethylpimilimidate (Harlow and Lane, 1988). Lysate was prepared as described in Carpenter and Bastiani (1991). Lysate was passed slowly (5-10 ml/hour) over the column. The column was then washed in 3-5 column volumes of the following buffers all containing protease inhibitors (20 μg/ml phenylmethanesulfonyl fluoride, 1 μg/ml antipain, chymostatin, leupeptin, pepstatin, N-p-tosyl-L-lysine chloromethyl ketone and N-tosyl-L-phenylalanine chloromethyl ketone): (1) 10 mM triethanolamine (TEA) pH 8.2, 0.15 M NaCl, 1% NP-40, (2) 10 mM TEA pH 8.2, 0.15 M NaCl, (3) 10 mM TEA pH 8.2, 0.15 M NaCl, 0.5% deoxycholic acid (Sigma), (4) 10 mM TEA pH 8.2, 1.0 M NaCl, 1% NP-40. The column was eluted at pH 11.5 in elution buffer (50 mM TEA pH 11.5, 0.15 M NaCl, 1% NP-40, protease inhibitors). Eluate was collected in 1 ml fractions, precipitated with trichloroacetic acid (TCA) and analyzed using SDS-PAGE.

Generation of serum antibodies

TCA-precipitated column fractions containing the 38×10^3 M_r doublet were used to generate serum antibodies. Dried protein was resuspended in 50-100 μl PBS, emulsified with an equal volume of Freund's adjuvant and injected intraperitoneally into a BALB/c female mouse. The first injection used Freund's complete adjuvant and the three subsequent injections (at two-week intervals) used Freund's incomplete adjuvant. Serum was collected one week after each injection and used to label grasshopper embryos as above.

Protein sequencing

Because Lachesin appears to be blocked to sequencing, we generated peptide fragments for sequencing. Approximately 30 μg of protein was run on a 12.5% preparative polyacrylamide gel that had been pre-run 1 hour. The gel was stained 5 minutes in 0.3 M $CuCl_2$, washed 2× 2 minutes in distilled water and photographed on a dark background (Harlow and Lane, 1988). The protein band was cut out and washed 3× 10 minutes in 0.25 M Tris-HCl pH 9, 0.25 M EDTA. After a 1 hour wash in distilled water, the gel was cut into 1 mm pieces and dried under vacuum in a 1.6 ml microfuge tube. The gel was rehydrated in a solution containing 0.1 μg/μl sequencing grade Trypsin (Boehringer Mannheim) in 200 mM NH_4HCO_3 pH 7.5 and incubated overnight at 37°C. The peptides were eluted from the gel 24 hours in 1 ml of 200 mM NH_4HCO_3 pH 7.5, then 4 hours in 0.5 ml 200 mM NH_4HCO_3 pH 7.5. The eluted solution was dried thoroughly in a speed vac. The sample was resuspended in 0.2% TFA (approx. 230 μl) and the peptides separated using a C-18 HPLC column. Eluted peaks were sequenced directly.

PCR primer design and amplification of Lachesin DNA sequences

Two of the peptide sequences obtained were used to design degenerate oligonucleotide primers for the polymerase chain reaction. We made degenerate, inosine-containing sense (5′ GAG AGA ATT CTI $^{A}/_{T}{}^{C}/_{G}$I AA$^{C}/_{T}$ AA$^{C}/_{T}$ CA$^{A}/_{G}$ CA$^{C}/_{T}$ TA) and antisense (5′ GAG AAT TCT CIG TGA A$^{T}/_{C}$T CGT CIG CIG T) primers corresponding to the ends of one of the fragments (#69) and amplified a DNA fragment of the expected size (60 bp) from grasshopper first-strand cDNA (Innis et al., 1990). Because the relationship of the tryptic peptides in the intact protein was not known, both sense (5′ GAG AGA ATT CGC I$^{C}/_{T}$T ICA $^{A}/_{G}$TA $^{C}/_{T}$A $^{C}/_{T}$AT GGA) and antisense (5′ GAG AGA ATT CTC CAT $^{G}/_{A}$TC $^{G}/_{C}$TA $^{T}/_{C}$TG IA$^{G}/_{A}$ IGC) primers were made for a region of another peptide (#81). The sense primer from peptide #81 and the antisense primer from peptide #69 amplified a 140 bp sequence. Both reactions used 34 cycles of: 94°C 30 seconds, 45°C 30 seconds, 72°C 1 minute. All primers contained the *Eco*RI restriction enzyme recognition sequence at their 5′ ends. The amplified fragments were subcloned into the *Eco*RI site of the Bluescript plasmid. Dideoxy sequencing revealed that both the 60 bp and 140 bp fragments encode the correct amino acid sequence.

Library screening and sequencing

The 140 bp polymerase chain reaction (PCR) fragment was labelled using an oligolabelling kit (Pharmacia) and used to screen a λgt11 library prepared by K. Zinn (Snow et al., 1988). 12 positive plaques were isolated from approximately 500,000 phage. The positive plaques were screened using the same PCR primers and conditions given above. 11 of the 12 gave fragments of the expected size. 8 of these contained an identical 2 kb insert that was subcloned and sequenced by dideoxy sequencing. The DNA and protein sequence data were analyzed using the GCG data analysis package (Devereux et al., 1984).

RESULTS

Lachesin is expressed by neuronal precursor cells during early embryogenesis

Lachesin is a very early marker of neuronal differentiation and persists only on cells that follow neuronal developmental pathways. Because Lachesin expression begins early and continues in changing patterns throughout development, we will take a chronological approach to describing its expression. At 30°C, grasshopper embryos hatch (100% of development) 20 days after the eggs are laid. Thus each day represents 5% of embryonic development. The embryo first becomes visible as a disc-shaped group of cells at 10% of development. The anterior region of the disc enlarges by 12-14% of development and will form the head. The posterior of the disc-shaped embryo elongates and will form the body of the embryo (see Fig. 1A). As the embryo elongates, gastrulation and segmentation occur in a temporal gradient from anterior to posterior (Bentley et al., 1979).

Fig. 1 gives an overview of Lachesin expression on neurogenic cells, neurons and axons throughout the first 35% of

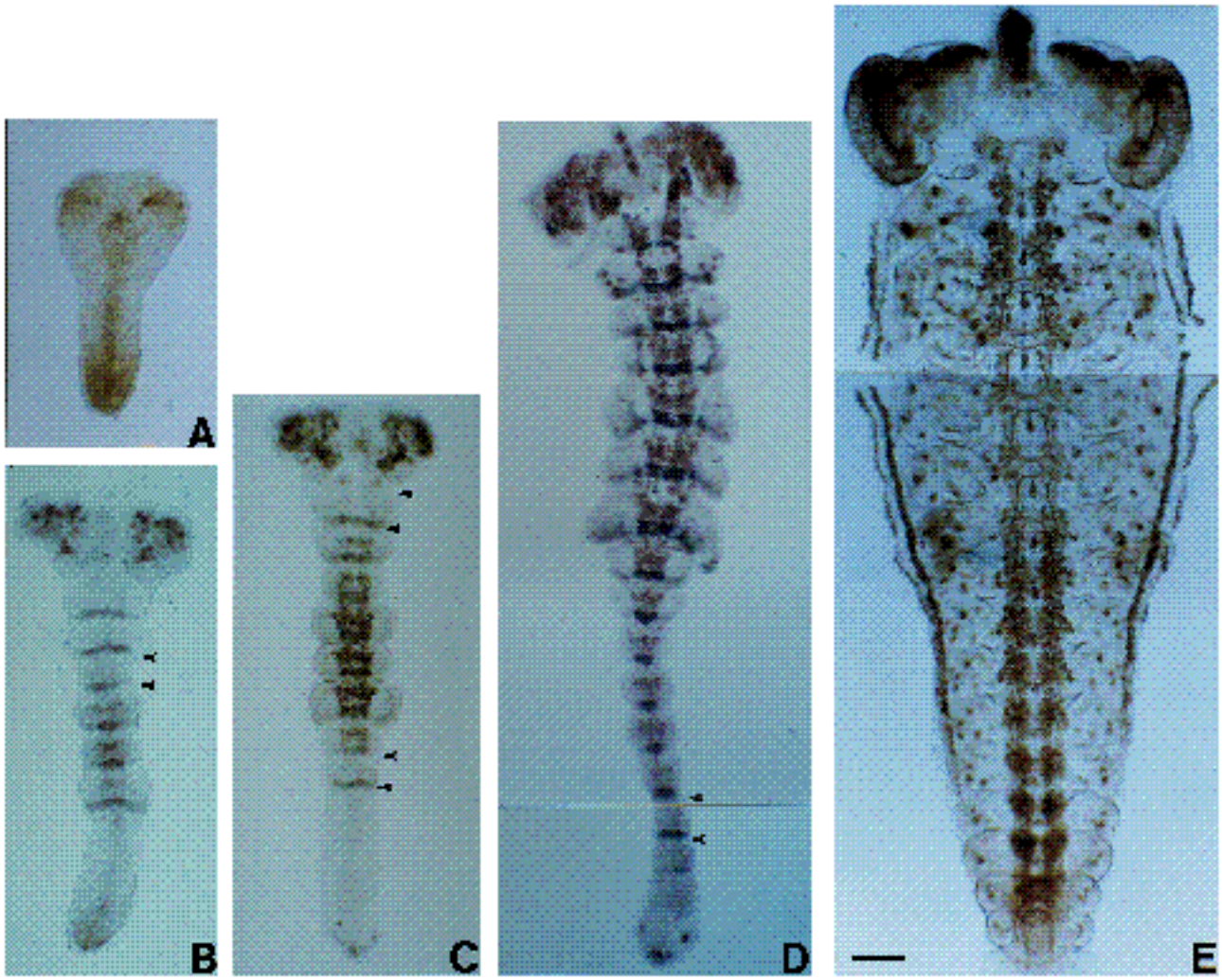


Fig. 1. Lachesin expression during early embryogenesis. All panels show labelling with the 1C10 mAb (brown). Embryos in B-D are double labelled with the anti-engrailed mAb (gray/black). Arrowheads in B-D delineate 'border' segments that express engrailed but not Lachesin. All panels are dorsal views except D which shows the ventral surface of the embryo. (A) At 15% of development, Lachesin is expressed by cells located at positions where the first neuroblasts will appear in the head region. Labelling is also seen in the proliferative zone at the posterior end of the embryo and light labelling is seen in gastrulating cells at the midline. (B) At 18% of development, Lachesin-expressing cells in the head region have divided to form clusters of neuroblasts and their progeny. Lachesin expression is beginning on differentiating neurogenic cells in the first segments to form, the thoracic segments. Engrailed expression defines the posterior margin of each developing segment and precedes Lachesin expression by less than 1% of development. (C) At 22% of development, Lachesin expression defines the neurogenic region. Lachesin-expressing cells in the thoracic segments have enlarged to form neuroblasts. Expression of Lachesin in each segment (here in the first abdominal (A1) and second subesophageal (S2) segments) continues to follow closely behind engrailed expression (in A2 and S1, arrowheads). (D) At 28% of development, engrailed expression has begun in half of segment A8 and is seen across A7 (arrowheads). Morphological segmentation in the mesoderm has begun in A6 or A7 and the first Lachesin-expressing cells are seen in A6. Lachesin-expressing cells can be seen in the developing limb buds in the three thoracic segments. These are the differentiating Ti1 and Cx1 sensory neurons. Differentiating sensory neurons can also be seen on each side of the tail region in the future cerci and laterally in the subesophageal segments, particularly in S1. (E) At 35% of development, axonogenesis has begun in the older segments (T1-3, S1-3, A1-4). The anterior and posterior commissures and longitudinal connectives are visible in segments S2-T3. Axon bundles in the developing eye also express Lachesin. Sensory neuron clusters express Lachesin in the body wall of all segments, with large bundles of cells appearing in A1 (the auditory organ) and in the developing mouth parts of S1. The median neuroblast bundle is apparent in each segment at the midline. Legs have been removed from this embryo. Scale bar: (A-D), 250 μm; (E), 300 μm.

embryonic development. Expression starts at approximately 15% of development in a few cells in the future brain and in the gastrulating midline and proliferative tail region (Fig. 1A). At 18% of development (Fig. 1B), many neuroblasts (NBs) and their progeny in the head region and a few neurogenic cells in the future thoracic segments express Lachesin. At 22% of development (Fig. 1C), Lachesin expression defines the entire neurogenic region in the thoracic segments and is expressed by ventral neurogenic ectoderm as well as NBs and their first progeny. Non-neu-

rogenic ectoderm shows no Lachesin expression. Later, the first sensory neurons in the periphery express Lachesin as they begin to differentiate in the limb buds and future cerci at approximately 28% of development (Fig. 1D). At 35% of development (Fig. 1E), Lachesin is expressed by NBs, ganglion mother cells, neurons and growing axons in the central nervous system (CNS), retinal neurons and axons in the developing eyes, and developing sensory structures in the body wall and limbs.

Lachesin is expressed on differentiating neuronal cells before they are otherwise distinguishable from their neighbors. The first Lachesin-expressing cells in the developing brain (Fig. 1A) appear to be the first neural cells to differentiate in the embryo and will form neuroblasts. Clusters of neuroblasts and their progeny can be clearly seen in the head by 18% of development (Fig. 1B). Lachesin expression also precedes morphological changes associated with neuronal differentiation in the neurogenic region of each segment (Fig. 1B-D) and in the developing limbs (Fig. 4B).

Lachesin expression predicts the position of neuronal differentiation in each developing segment. A single cell on each side of the midline begins to express Lachesin just as the mesoderm first displays morphological signs of segmentation (not shown). Neuroblasts first appear and delaminate from the ventral ectoderm at the site of the first Lachesin-expressing cells, which are initially the same size as other cells in the ventral layer. These first Lachesin-expressing cells may themselves become the first neuroblasts, or alternatively they may mark the position where the first NB appears. In either case, early Lachesin expression identifies the first site of neuronal differentiation. Lachesin expression in a segment quickly (within another 1% of development) expands to include a row of 4 or 5 cells that span the midline at this same anterior-posterior position, then expands to include a rectangle of cells in the posterior two thirds of each segment (Fig. 1C). Within 2% of its first appearance, Lachesin is expressed throughout the neurogenic region (Fig. 1C,D).

To determine the timing of Lachesin expression in relation to segmentation, we compared the onset of Lachesin expression to that of the engrailed protein, an early molecular marker of segmentation. Lachesin expression appears just after the first expression of the engrailed protein, as indicated by double labelling (Fig. 1B-D). Engrailed is expressed in cells that will form the posterior border of developing segments just before morphological segmentation begins in the mesoderm (Patel et al., 1989). Lachesin first appears within 1% (5 hours) of the first engrailed expression, just as the mesoderm begins morphological segmentation.

In addition to intense labelling associated with Lachesin expression on individual cell surfaces, more diffuse labelling is seen at early developmental stages. At 15% of development, as gastrulation is beginning in the future thoracic regions, diffuse labelling is seen in the mesoderm and in the proliferative zone at the posterior end of the embryo (Fig. 1A). At the time that Lachesin is seen at the position of the first future neuroblasts, a more diffuse stripe of labelling is seen throughout the posterior two thirds of the segment, in both ectodermal and mesodermal layers. In general, this labelling appears to be most intense in the middle of each segment and trails off toward the segment edges. As neurogenesis continues, this diffuse labelling is lost and Lachesin becomes strictly restricted to the central region of the embryo, the neurogenic ectoderm (Fig. 1B,C). Expression is absent in the lateral body wall ectoderm until the differentiation of sensory neurons in the peripheral nervous system (PNS; see below).

In summary, Lachesin is first expressed at positions where neuroblasts appear in the head and in each developing segment. Lachesin is expressed soon after engrailed is expressed in the posterior cells of each segment, indicating neuronal differentiation begins immediately after the onset of segmentation. As neurogenesis proceeds, Lachesin expression becomes restricted to the central part of the embryo, the neurogenic ectoderm.

Lachesin expression continues on neuroblasts but is lost on non-neuronal cells

Early, all cells in the neurogenic region express Lachesin (Figs 1C-E, 2A). These include ventrally located neurogenic cells (Fig. 2A) as well as cells in the more dorsal neuroblast layer (Fig. 2B). As neuroblasts continue to segregate from the ventral ectoderm they express Lachesin (Fig. 2A, asterisks). In contrast, Lachesin expression is lost in neuroectodermal cells that do not become neuroblasts. These include the sheath cells that surround each neuroblast (areas between neuroblasts in Fig. 2B-D).

All 30 neuroblasts in each hemisegment express Lachesin (Figs 1D, 3A), as do neuroblasts in the future brain (not shown). The specific order of appearance of the 30 NBs within each hemisegment based on Lachesin expression is somewhat different from that previously described (Doe and Goodman, 1985a). The first Lachesin-expressing cells appear in the position of future NB 3-5 (not shown). Expression is next seen at the future NB 4-1 position and in a stripe between these two cells. In agreement with our observations, Doe and Goodman saw NB 3-5 appear first. However, they next saw the appearance of NB 2-5, followed by a group of 8 NBs. Cells in the position of row 2 do not express Lachesin until many NBs in the posterior two thirds of the segment have begun to differentiate. Doe and Goodman based the birth of a NB on the appearance of the first ganglion mother cell. Since Lachesin appears to be an early molecular marker of neuronal differentiation, it is possible that Lachesin allows us to document the onset of NB differentiation. Different rates of NB development could then explain the discrepancy between the two methods for documenting NB birth.

In both grasshopper and fly, neuroblasts divide asymmetrically to produce a series of ganglion mother cells that then undergo a terminal division to form neurons. 1C10 mAb labelling clearly reveals this stem cell division pattern (Fig. 2B-D). Within a family, Lachesin expression fades in the NB and becomes more intense in ganglion mother cells and neurons (Fig. 2D). Later in development, only some NB families label. Because of the large number of labelling neurons at later ages, it is difficult to determine whether these late labelling neuroblasts are the same neuroblasts that have been reported to divide late in locust development (Shepherd and Bate, 1990). However, in general, fewer NBs express Lachesin at later ages than are reported to be

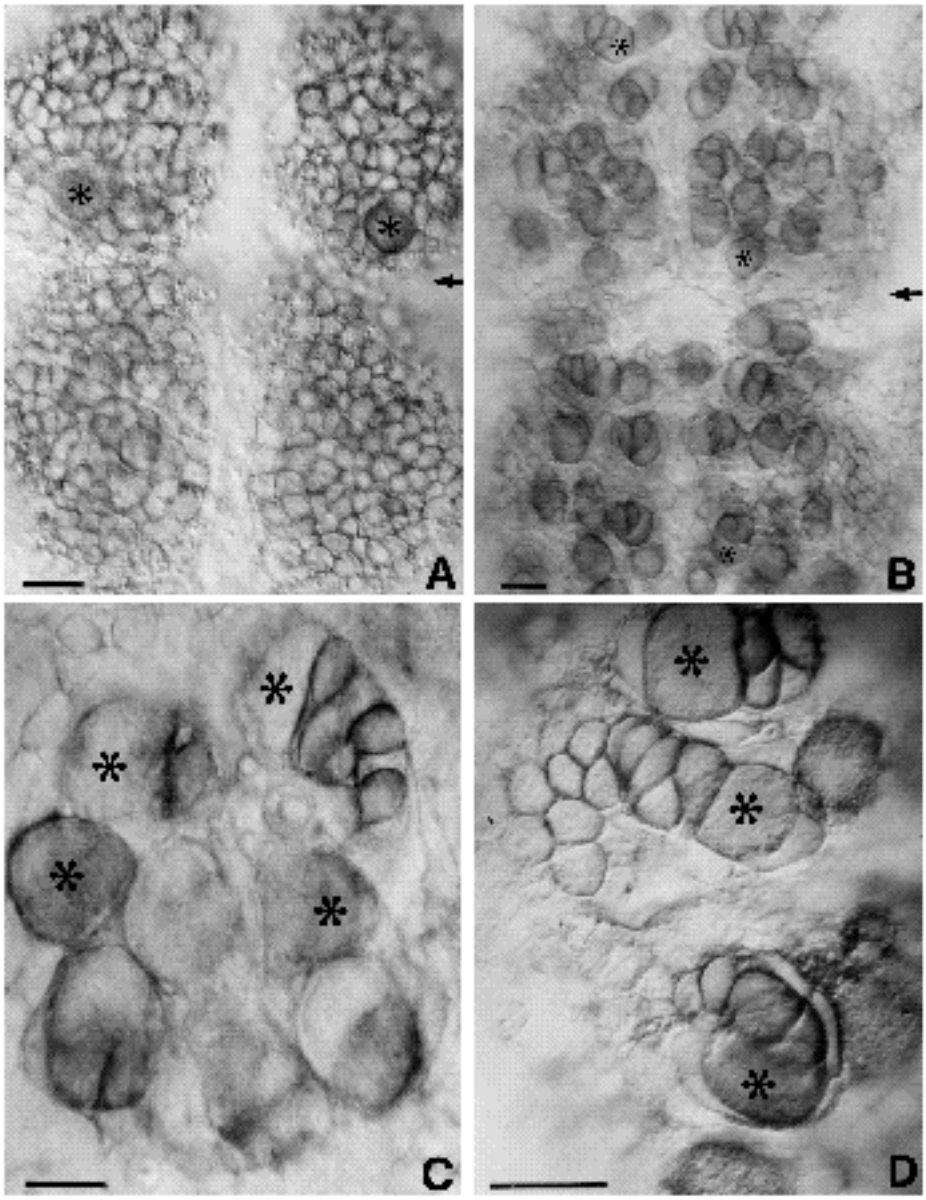


Fig. 2. Ventral view of lachesin expression during neurogenesis. (A) Segments T1 and T2 at 30% of development. Neuroepithelial cells transiently express Lachesin. Neuroblasts (*) label strongly as they are segregating from the neuroepithelium. Arrow indicates segment boundary. (B) Two 38% segments. Neuroblasts divide asymmetrically to produce chains of ganglion mother cells that divide to form neurons. These families express Lachesin. Lachesin expression is lost in the surrounding cells that are becoming non-neuronal. (C) Higher magnification of a 38% neuroblast family. Lachesin expression has begun to fade in some of the neuroblasts. (D) By 55% neuroblast, families are quite extensive and continue to express Lachesin. Scale bars: 25 μm.

dividing. The median neuroblast stops expressing Lachesin at approximately 50% of development, long before it is reported to stop dividing and die (at 75% of development; Shepherd and Bate, 1990). Thus the time at which a neuroblast stops expressing Lachesin does not seem to correlate with its death. Other factors such as intrinsic timing or local interactions may be responsible for the loss of Lachesin expression by a NB.

Lachesin is expressed by pioneer neurons and axons in the legs and by differentiating neuronal cells in the lateral ectoderm

Sensory neurons in the peripheral nervous system express Lachesin before they delaminate from the epithelia of the limbs, mouthpart rudiments and body wall (Figs 1D, 3A). The Ti1 neurons in the limb tips begin to express Lachesin at 28-30% of development while still in the limb epithelium from which they arise (Fig. 3B, arrowheads). The Ti1 neurons move into the limb and their axons pioneer the 5B nerve (Keshishian and Bentley, 1983). The pioneer growth cones and axons express Lachesin, as do the guidepost neurons Fe1 and Tr1 (Fig. 3C, arrows). Differentiating sensory neurons of the femoral chordotonal organ also express Lachesin (Fig. 3C, open arrow).

In the head, cells in the region of the optic medulla and lamina, areas that later receive inputs from retinal photoreceptors, form distinct layers and express Lachesin. Some neurons in the retina as well as bundles of photoreceptor axons also have Lachesin on their surfaces (Fig. 1E). Whether labelling cells in the retina constitute all photoreceptors or a subset has not been determined.

Sensory neurons differentiate from the body wall ectoderm throughout the embryo in a manner similar to the limb sensory neurons. Approximately 4% of development after ectodermal segmentation, individual cells begin to express Lachesin in the ectoderm. These cells will form the sensory organs found in the body wall of each segment (Fig. 1E). These sensory structures include the auditory organ in

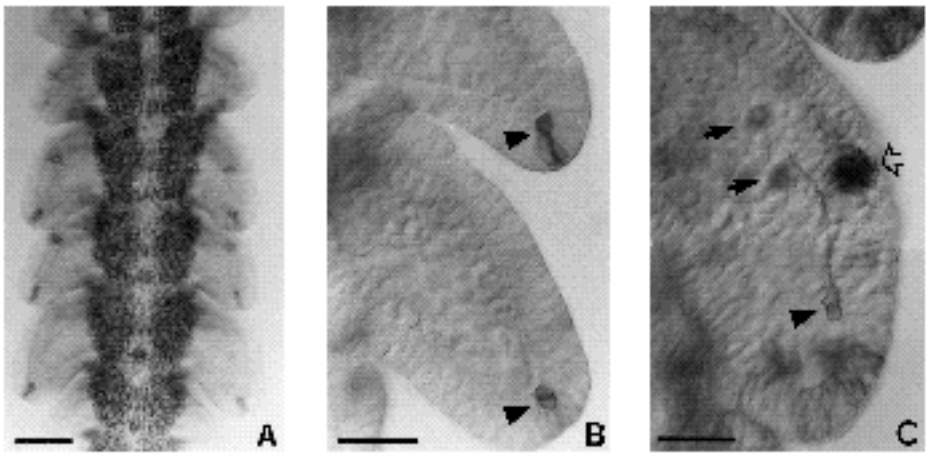


Fig. 3. Lachesin expression in the legs. (A) Ventral view of a 30% embryo. Neuroblasts in the central neurogenic region and differentiating sensory neurons in the limbs express Lachesin. (B) Second and third thoracic limbs at 30% of development. Differentiating sensory neurons at the limb tip (Ti1) express Lachesin as they segregate from the limb epithelium (arrowheads). (C) Second thoracic leg at 32% of development. The growth cones and axons of the Ti1 neurons that pioneer the axon pathway to the CNS express Lachesin. The guidepost cells Tr1 and Cx1 also express Lachesin (arrows), as do a large cluster of cells at the position of the femoral chordotonal organ (open arrow). Scale bars: (A) 250 μm; (B,C) 100 μm.

segment A1, pleural chordotonal organs and sternal chordotonal organs in segments A2-A8, and sensory cells of the dorsal and ventral clusters in all segments (Meier et al., 1991).

A cluster of cells on each side of the tail begin to express Lachesin at 22-23% of development, long before segmentation reaches the tail at approximately 30% of development. The labelling cells are in the region that will form the cerci and may be differentiating as cercal sensory structures (Fig. 1D). This early expression of Lachesin in the tail region suggests that the caudal end of the animal acquires a distinct segmental identity before segmentation is complete in the abdomen. Segmentation in grasshopper, a short germ band insect, may thus be a filling-in process rather than solely an anterior-to-posterior progression. The definition of a distinct terminal segment before segmentation is complete in the more anterior abdominal segments may provide a mechanism for regulating segment number by defining a caudal limit to segmentation. Cells in the proctodeum, or hind gut rudiment, also label before abdominal segmentation is complete, at approximately 22% of development. By 27% these cells form an epithelium that continues to label with the 1C10 mAb.

Lachesin is expressed by neurons and axons as they arise, but becomes restricted to a subset of neurons and axons later in development

At 40% of embryonic development the 1C10 mAb labels neurons and most or all axons in the grasshopper embryo. Fig. 4A shows a dorsal view of three segments in a 40% embryo labelled with the 1C10 mAb (see also Fig. 6A). All axonal pathways appear to express Lachesin. In the CNS,

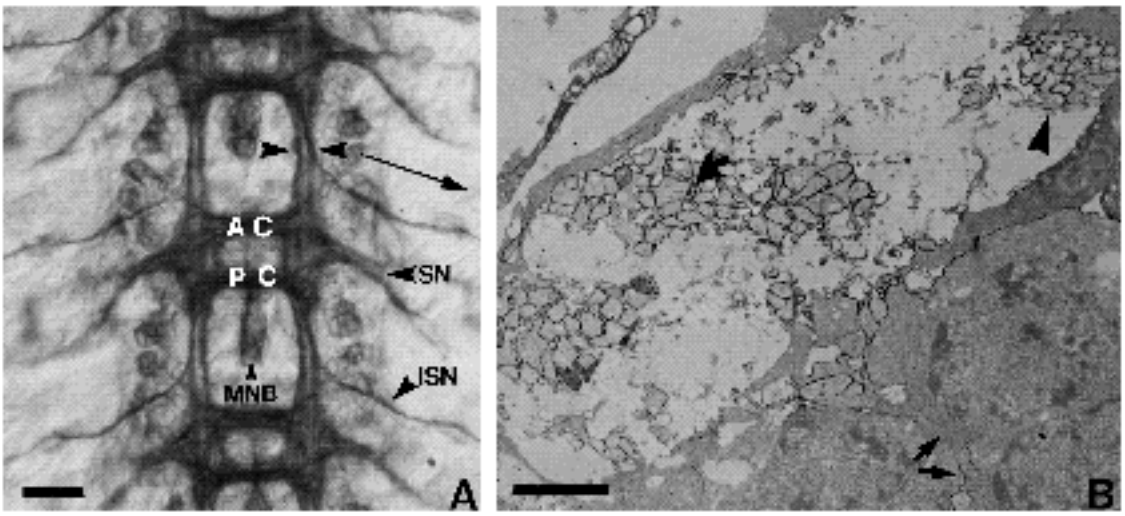


Fig. 4. Lachesin is present on growing axons. (A) Dorsal view of three segments in a 40% embryo. Lachesin is expressed on the axonal scaffold and on nerve cell bodies. At this stage, Lachesin appears to be expressed on all axons of the longitudinal connectives, the anterior commissures (AC), posterior commissures (PC), the segmental nerves (SN) and intersegmental nerves (ISN). Median neuroblasts (MNB) and their families of ganglion mother cells and neurons also label strongly. (B) Electron micrograph taken at the level of the two arrowheads in A. The lateral bundle of axons (arrowhead) leaves the CNS forming the intersegmental nerve. Lachesin is present on axons and filopodia (curved arrow), and is expressed discontinuously on nerve cell bodies (arrows). Scale bar: (A), 60 μm; (B), 5 μm.

these axons make up the longitudinal connectives that connect segmental ganglia, as well as commissural bundles within each ganglion. In the PNS, axons of the segmental and intersegmental nerves are labelled. The bundle of midline cells arising from the median neuroblast in each segment labels strongly. 1C10 mAb labelling of living embryos in the same pattern indicates Lachesin is present on cell surfaces (not shown).

Most or all neurons express Lachesin as they differentiate. We have followed several identified neurons from their birth to determine which cells express Lachesin. All median precursor neurons as well as the Q1, Q2, G and C neurons express Lachesin when they first appear. The corner cells, which migrate to their final positions from the next posterior segment, either do not express Lachesin or express such low levels of Lachesin that we cannot clearly identify these cells during their migration. Double labelling with an antibody to even skipped, an antibody that labels the corner cells as they are born and migrate (Patel et al., 1992), may allow us to determine whether these migrating neurons express Lachesin. Later in development only a subset of neurons continue to express Lachesin.

Fig. 4B is an electron micrograph taken at the position indicated by the arrowheads in Fig. 4A. The lateral bundle of axons (Fig. 4B, arrowhead) exits the CNS, forming the intersegmental nerve. It appears all axons in these bundles are labelled. Growth cones express Lachesin from the time they emerge from cell bodies (not shown). Filopodia label intensely, particularly where they are found in aggregates (Fig. 4B, curved arrow). In some places, labelling is seen throughout enclosed spaces between axons. The electron micrograph confirms that the 1C10 mAb is recognizing a surface antigen. Labelling of cell bodies is often discontinuous, as seen in Fig. 4B (arrows).

Lachesin expression becomes restricted to a subset of commissural and longitudinal axon fascicles by 60% of development (not shown). No commissural expression is seen in hatchling ganglia and only a few longitudinal fascicles, one of which is a ventral bundle of sensory axons, continue to express Lachesin. The 1C10 mAb labels the perineural glial layer from around 70% into the adult (not shown).

Biochemical characterization and purification of Lachesin

The 1C10 mAb immunoprecipitates a 38×10^3 M_r protein from embryonic membranes (Fig. 5A lane 1). The 3B11 mAb, which recognizes the 70×10^3 M_r Fasciclin 1 protein (Fig. 5A lane 2), served as a positive control (Bastiani et al., 1987). Non-specific binding to the antibody complex is seen at the bottom of both lanes. The precipitated antigen has been resolved as a doublet on higher percentage polyacrylamide gels (not shown), which is consistent with the results seen by affinity purification and western blot analysis (see below).

A 38×10^3 M_r doublet was purified from grasshopper embryonic lysates by mAb affinity chromatography. Fig. 5B shows a Coomassie-stained polyacrylamide gel of a purified column fraction. Whole column fractions were used to generate polyclonal Ab in a mouse. The serum antibody (SAb) generated labels grasshopper embryos in the same pattern as the original mAb (not shown). Further, this serum

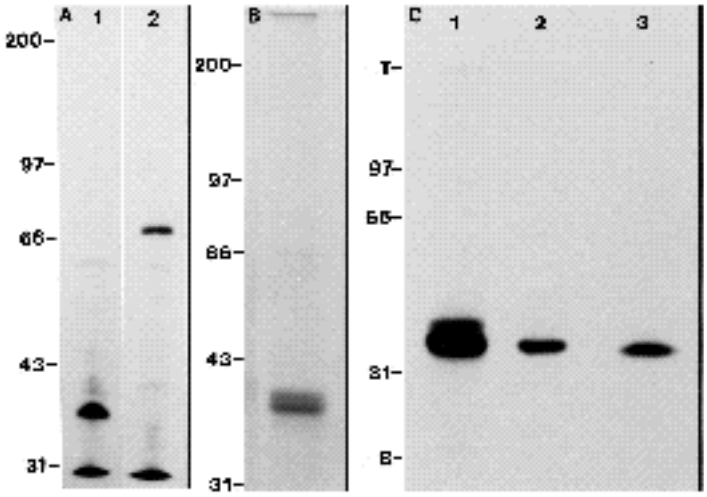


Fig. 5. Isolation and purification of lachesin.
(A) Immunoprecipitation. ^{125}I-labelled membrane proteins were precipitated with the (1) 1C10 and (2) 3B11 mAbs and run on a 7.5% polyacrylamide gel. The 1C10 mAb precipitates a 38×10^3 M_r protein. The 3B11 mAb precipitates the 70×10^3 M_r Fasciclin 1 glycoprotein. (B) Immunoaffinity purification. The 38×10^3 M_r Lachesin protein was purified from grasshopper embryonic lysate using a 1C10 mAb affinity column and run on a 10% polyacrylamide gel. Purified protein was used to generate serum antibodies and for protein sequencing.(C) Western immunoblot using 1C10 mAb and polyclonal Ab to Lachesin on proteins transferred from a 10% polyacrylamide gel. Lane 1. Embryonic membrane proteins run under reducing conditions and probed with anti-Lachesin serum antibody. Lane 2. Embryonic membrane proteins run under reducing conditions and probed with the 1C10 mAb. Lane 3. Embryonic membrane proteins run under non-reducing conditions and probed with the 1C10 mAb. The doublet seen with the serum antibody results from cleavage of a glycosyl phosphatidyl inositol moiety from the Lachesin protein.

recognizes a 38×10^3 M_r doublet on a western immunoblot (Fig. 5C lane 1). We conclude that the purified 38×10^3 M_r protein is responsible for the labelling pattern seen with the 1C10 mAb and is therefore the Lachesin protein. The mAb detects only the lower band on this western immunoblot (Fig. 5C lanes 2 and 3), longer exposure of the blot revealed small amounts of the upper band of the doublet. The lower band migrates identically under reducing (lane 2) and non-reducing (lane 3) conditions.

The relationship between the two bands of the doublet has been determined by treating embryos with phosphatidyl inositol-specific phospholipase C (PI-PLC). PI-PLC treatment eliminates 1C10 mAb labelling (compare Fig. 6B and A). Further, when analyzed by western immunoblot using the 1C10 mAb (Fig. 6C) Lachesin is released to the supernatant (S) of PI-PLC-treated embryo cultures while it remains with the membranes (M) of untreated embryos. The change in protein mobility within a polyacrylamide gel is consistent with the loss of a lipid tail after PI-PLC treatment seen with Fasciclin 1 (Hortsch and Goodman, 1990). Phosphatidylcholine-specific phospholipase C did not affect 1C10 mAb labelling. We conclude from these experiments that grasshopper Lachesin is bound to the membrane by a glycosyl phosphatidylinositol anchor.

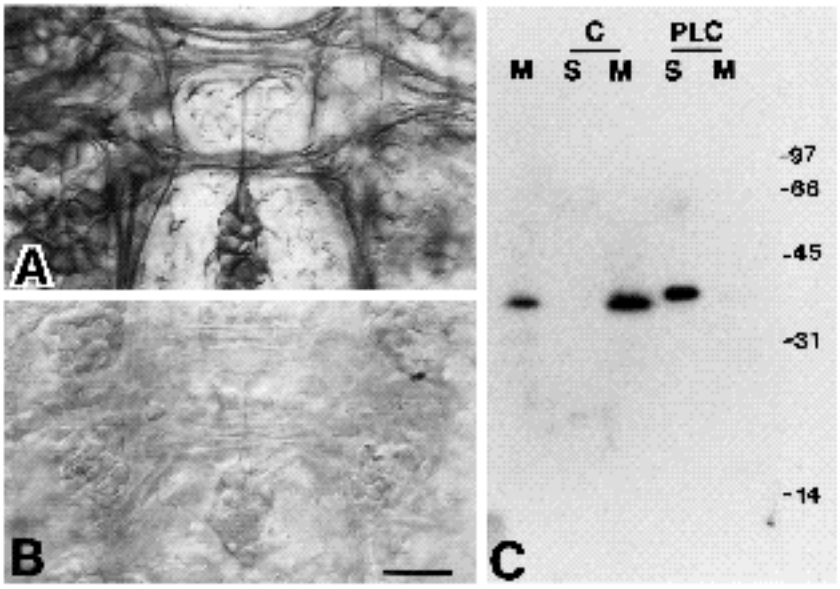


Fig. 6. Lachesin is linked to the membrane through a glycosyl phosphatidylinositol anchor. (A) 1C10 mAb labelling in one segment of an untreated embryo at 40% of development. Axons of the commissures, longitudinal connectives and median fiber tract as well as nerve cell bodies are labelled. (B) Labelling in an embryo treated with 1.5 U/ml PI-PLC for 2 hours. Lachesin is no longer seen on the axons or cell bodies of the CNS. (C) Western immunoblot of PI-PLC experiment. Left to right: Standard membrane preparation (M). Membrane preparation (M) and supernatant (S) from 10 embryos cultured 2 hours in the absence of PI-PLC. Membrane preparation (M) and supernatant (S) from 10 embryos cultured 2 hours in the presence of PI-PLC. All Lachesin has been removed from the membranes of PI-PLC-treated embryos and is present in the supernatant. The change in protein mobility on a polyacrylamide gel is consistent with the removal of a glycosyl phosphatidyl inositol moiety (see text). Scale bar: 40 μm.

The protein doublet seen by both immunoprecipitation and affinity purification must be due to the presence of two forms of Lachesin, one with and one without a PI tail. This may indicate that endogenous phospholipases are present in the grasshopper embryo. Older protein preparations seemed to contain more of the higher, PI-cleaved species. The fact that PI-PLC cleavage exposes a new epitope may explain why the SAb is more sensitive to the upper band (Fig. 5C lane 1). The SAb was able to detect both species in the western shown in Fig. 5C while the mAb only detected the more prevalent lower band.

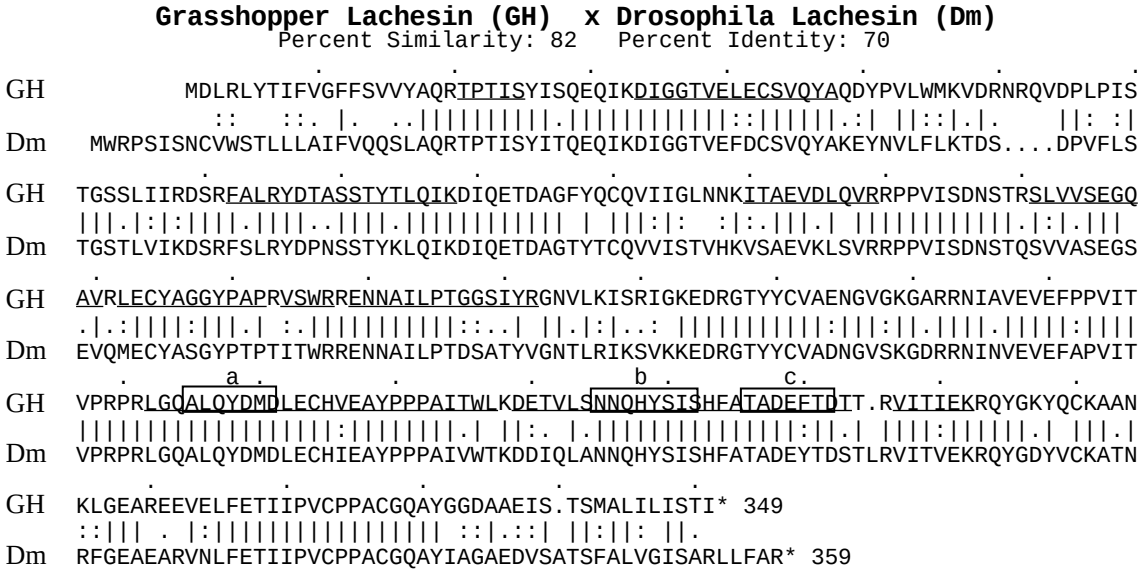
Molecular characterization of the *Lachesin* gene

In order to characterize the Lachesin protein further, we cloned the *Lachesin* gene. Since *Lachesin* was N-terminally blocked to protein sequencing, we generated and sequenced tryptic fragments. This peptide sequence was used to design degenerate oligonucleotides to amplify the grasshopper sequence from first-strand cDNA by PCR. Two PCR fragments of 120 bp and 60 bp contained coding sequence that matched peptide sequence outside the regions used to design the primers. The larger fragment was used to screen an embryonic grasshopper lambda gt11 cDNA library. Twelve positive cDNAs were isolated after screening approximately 500,000 phage. Eleven of these phage yielded the correct PCR fragments using both primer sets. Eight of these isolates contained an identical 2 kb insert, one of which was sequenced.

The cDNA contains an open reading frame encoding a 349 amino acid protein with a predicted relative molecular mass of 38,045. Peptide sequence from tryptic fragments of the purified protein match the sequence in the open reading frame, indicating we have cloned the grasshopper *Lachesin* gene. Underlined amino acid sequences in Fig. 7A are those found by peptide sequencing. The open reading frame contains an N-terminal hydrophobic domain characteristic of a signal sequence for secreted or membrane-bound extracellular proteins (von Heijne, 1985). The protein sequence contains no potential transmembrane domains, but does contain a hydrophobic C-terminal sequence characteristic of proteins that are linked to the membrane through glycosyl phosphatidyl inositol (GPI) anchors (Ferguson and Williams, 1988; Cross, 1990; Moran and Caras, 1991). This is consistent with our biochemical data for grasshopper Lachesin.

Initial comparisons to the DNA sequence data base indicated grasshopper Lachesin was similar to the *Drosophila* amalgam protein (Seeger et al., 1988). In order to determine whether Lachesin was the grasshopper homolog of amalgam, we used the same PCR primers to amplify sequences from *Drosophila* cDNA. Products of the expected size were amplified and were verified by DNA sequencing. The PCR fragment was then used to isolate a *Drosophila* Lachesin cDNA clone. Sequence comparisons showed that the isolated *Drosophila* cDNA is not amalgam and is remarkably similar to grasshopper Lachesin (Fig. 7A). Based on the high degree of sequence similarity we conclude that we have isolated the *Drosophila* homolog of grasshopper Lachesin. When the three Ig domains which make up 90% of the proteins are compared, the predicted Lachesin amino acid sequences are 75% identical. This is much higher than the identities seen between other genes that have been cloned in both grasshopper and *Drosophila* such as *Fasciclin 1* (50% identical; Zinn et al., 1988), *Fasciclin 2* (41%; Harrelson and Goodman, 1988; Grenningloh et al., 1991), *neuroglian* (65%; Bieber et al 1989; Grenningloh and Rehm, unpublished data) and *even skipped* (38%; Frasch et al., 1987; Patel et al., 1992). Considering the large evolutionary distance separating grasshopper and *Drosophila* (on the order of 300 million years), the high degree of sequence conservation indicates that a large portion of the Lachesin sequence may be important for its function. The 1C10 mAb does not label *Drosophila* embryos, so we have not determined if the expression of *Drosophila* Lachesin is similar to grasshopper Lachesin. While we do not know whether *Drosophila* Lachesin is also linked to the membrane by a GPI anchor, the presence of a C-terminal hydrophobic

A



B

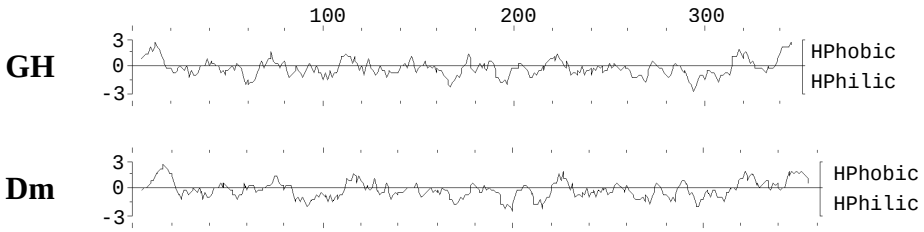


Fig. 7. Protein sequence in grasshopper and fruit fly. (A) Comparison of deduced amino acid sequence from grasshopper (GH) and *Drosophila* (Dm) Lachesin cDNA clones using the GCG BESTFIT program. Lines indicate amino acid identities, double dots indicate similar amino acids. Peptide sequences generated from tryptic fragments of purified grasshopper Lachesin are underlined. Sequences in boxes were used to design degenerate oligonucleotides for PCR amplification of both grasshopper and *Drosophila* Lachesin cDNA sequences. (B) Kyte and Doolittle hydropathy plots of grasshopper and *Drosophila* Lachesin showing nearly identical profiles. Both plots show a hydrophobic N-terminal signal sequence and a C-terminal hydrophobic domain characteristic of proteins attached to cell membranes by a GPI anchor. The *Drosophila* and grasshopper Lachesin sequences have been registered with Genbank and given the following accession numbers: DROLACH, L13255 and GHLACH, L13256.

domain characteristic of GPI-linked proteins suggests that it is (Fig. 7B).

Lachesin is a novel protein that belongs to the Ig superfamily

We compared the two Lachesin protein sequences to the sequence database using the TFASTA program (Devereux et al., 1986). Lachesin is most similar to a series of cell surface molecules containing the regularly spaced cystine residues characteristic of the immunoglobulin domain. Closer examination of the Lachesin sequence reveals three domains of internal homology corresponding to one V-type and two C-2-type Ig domains (Williams and Barclay, 1988). Lachesin is most similar to *Drosophila* amalgam, being 38% identical and 58% similar when conservative amino acid substitutions are tabulated. Amalgam is present on cells in the mesoderm and developing nervous system in *Drosophila* embryos, but its function is still unknown (Seeger et al., 1988). After amalgam, Lachesin is most similar (approximately 25% identity) to a group of Ig superfamily proteins in vertebrates and invertebrates. These include the vertebrate cell adhesion molecules L1 (Moos et al., 1988), TAG-1 (Furley et al., 1990), Ng-CAM (Burgoon et al., 1991), Bovine Opioid Binding Cell Adhesion Molecule (OBCAM; Schofield et al., 1988), the human colorectal tumor suppressor (DCC) protein (Fearon et al., 1990) and the insect L1 homologue, Neuroglian (Bieber et al., 1989). All of these proteins share Ig domains and all may function in adhesion or intercellular communication.

Lachesin also fits into a subgroup of proteins (Hellen et al., 1989) containing amalgam and the human poliovirus receptor (Mendelsohn et al., 1989) and OBCAM. These proteins all contain one variable and two constant Ig domains and share amino acid identities around the conserved cysteine residues. Fig. 8 shows an alignment of the two Lachesin proteins with the most similar proteins and the poliovirus receptor. Many of these molecules play a role in cell surface recognition events.

DISCUSSION

We have described the developmental expression and molecular characterization of Lachesin, a novel Ig superfamily molecule in grasshopper and fruit fly. There are

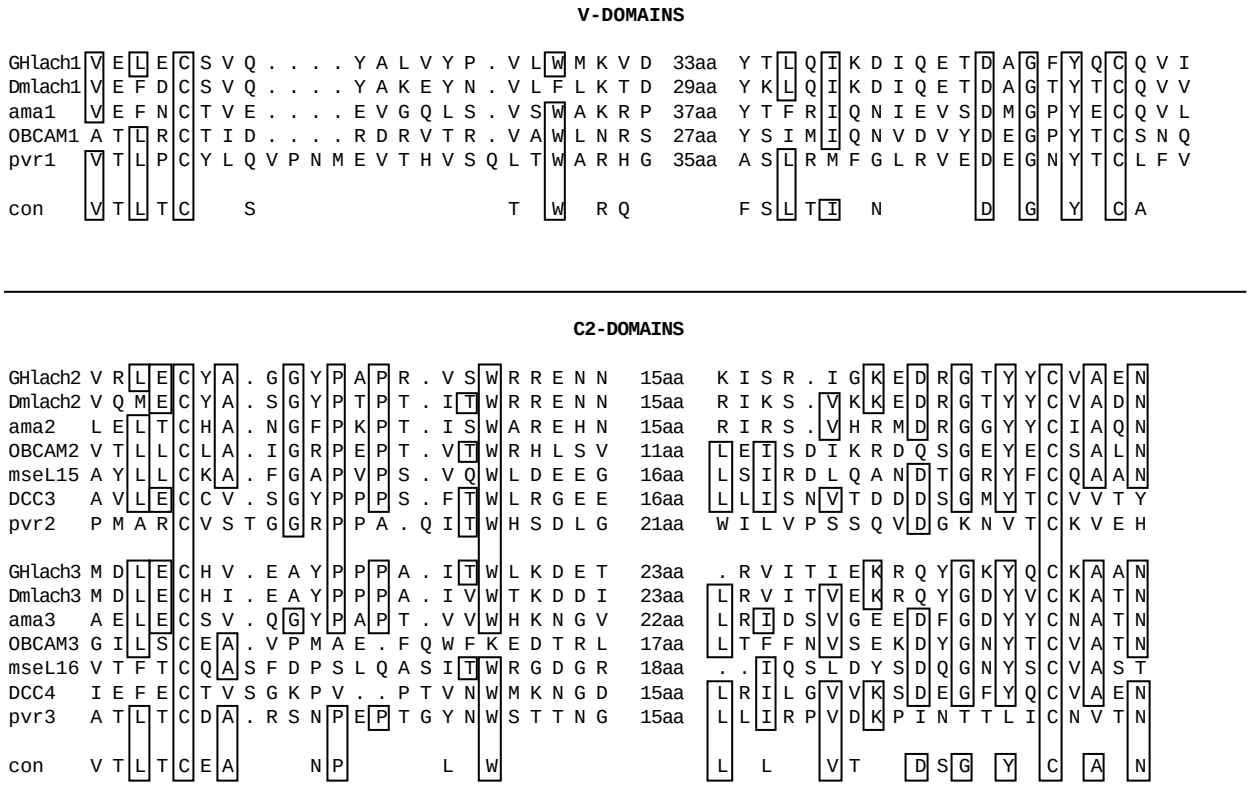


Fig. 8. Alignment of grasshopper and *Drosophila* Lachesin with other Ig superfamily Ig domains. Lachesin sequences were compared to the sequence data base and aligned using the GCG TFASTA program. All significant matches were to Ig domain containing proteins. Top shows alignment of V-domains and bottom shows the two C-domains of grasshopper Lachesin (GHlach), *Drosophila* Lachesin (Dmlach), *Drosophila* amalgam (ama), bovine opioid binding cell adhesion molecule (OBCAM), mouse L1 (mse L1) and the human colorectal tumor suppressor (DCC) and poliovirus receptor (pvr). con is a consensus of amino acids in Ig proteins taken from Williams and Barclay (1988). Boxed amino acids are those seen in at least four (V domains) or six (C domains) of the aligned proteins including the consensus sequence.

several indications that Lachesin plays an important role in insect development. First, the precisely regulated window of Lachesin expression follows groups of cells that are differentiating as neurons. Cells of the neurogenic ectoderm that do not follow neuronal developmental pathways lose Lachesin on their surfaces when they begin to differentiate as non-neuronal cells. Second, Lachesin is expressed very early in development. As one of the first surface molecules expressed by neurogenic cells, Lachesin may be involved in cell surface interactions at the beginning of neurogenesis. Third, the Lachesin protein has been highly conserved through the approximately 300 million years of independent evolution separating fruit fly from grasshopper. Fly and grasshopper Lachesin are 75% identical throughout their three Ig domains, indicating strong selective pressure for sequences throughout the molecule.

Lachesin is distinct from other insect molecules present during early neurogenesis. Because of its expression throughout the neurogenic region, *Lachesin* can be considered a pan-neural gene. Other pan-neural genes encode proteins found inside cells. *deadpan* (*dpn*) encodes a helix-loop helix protein expressed in neuroblasts, which is also involved in sex determination in *Drosophila* (Younger-Shepherd et al., 1992). *Lachesin* expression is in some ways similar to the pro-neural genes of *Drosophila*, which encode proteins that have been localized to the nuclei of neuroectoderm cells and neuroblasts. These genes include the genes of the *achaete-scute* complex and act to promote neurogenesis (reviewed in Ghysen and Dambly-Chaudiere, 1989). Lachesin expression is distinct from achaete (ac; Skeath and Carroll, 1992) and lethal of scute (l'sc; Martin-Bermudo et al., 1991), as these proteins are restricted to subsets of neurogenic cells. Nonetheless, the *Lachesin* gene could be a target for regulation by a combination of nuclear pan-neural and pro-neural genes and may thus be a downstream element in the molecular pathway that causes certain cells to acquire neuronal potential.

Several cell surface proteins are expressed at the earliest stages of neurogenesis as neurogenic cells segregate from non-neuronal ectoderm. These molecules are expressed in patterns distinct from Lachesin. These surface molecules are either expressed throughout neuronal and non-neuronal ectoderm (Notch, Johansen et al., 1989; Delta, Kopczynski and Muskavitch, 1989; big brain, Rao et al., 1990; fasciclin II, Harrelson and Goodman, 1988), or they are expressed in subsets of cells within the neurogenic region (Fasciclin 3, Patel et al., 1987; Grenningloh et al., 1990 and Dtrk, Pulido et al., 1992). Amalgam, the Ig molecule with the highest degree of sequence similarity to Lachesin, is seen in the mesoderm and developing CNS, but appears to be secreted

by non-neuronal tissue (Seeger et al., 1988 and M. Seeger and T. Kaufman, personal communication).

How might Lachesin function during segregation of the neurogenic ectoderm? The Ig domain structure of Lachesin suggests that it may act as an adhesion molecule. Lachesin may act as neuronal 'glue' and thus contribute to adhesive interactions that physically define the neurogenic region. The close juxtaposition of cells within a region of the embryo and the formation of distinct tissue borders may in turn influence the differentiation of cells within the neurogenic region. Adhesion molecules have been shown to play such a role in early neurogenesis in vertebrates. In *Xenopus*, misexpression of the adhesion molecule N-cadherin can disrupt early neurogenesis by creating inappropriate tissue borders (Detrick et al., 1990). Alternatively, Lachesin could directly function in cell signaling events that initiate neuronal differentiation. Other GPI-linked proteins have been implicated in cell signaling events. The GPI-linked neuronal protein F3/11 mediates cell repulsion caused by the extracellular matrix molecule J1-160/180 (Pesheva et al., 1993) and binding of ligands or antibodies to GPI-linked proteins can activate leukocytes (Robinson, 1991).

In the next major event after the segregation of the neurogenic ectoderm, some cells within the region enlarge and become neuroblasts while others become non-neuronal. These neuroblasts inhibit their neighbors from adopting neuronal fates through cell-cell interactions (Doe and Goodman, 1985b). A number of *Drosophila* mutations have been isolated that cause all cells in the neurogenic region to differentiate along neuronal developmental pathways (reviewed in Campos-Ortega and Jan, 1991). At least three of these neurogenic genes, *Notch*, *Delta* and *big brain*, encode cell surface molecules. *Notch* is expressed by both neurogenic and non-neurogenic ectodermal cells (Johansen et al., 1989). Both *Delta* and *big brain* are transcribed by cells of the ventral ectoderm before NB delamination. As NBs are recruited from the ventral ectoderm, *Delta* (Kopczynski and Muskavitch, 1989) and *big brain* (Rao et al., 1990) are turned off in neuroblasts and are maintained in the ventral ectodermal cells.

Lachesin expression during NB segregation is distinct from these other molecules and is complementary to the expression of Delta and big brain. Lachesin continues to be expressed on NBs and their progeny but is lost on cells that remain in the ventral ectoderm while the reverse is true for Delta and big brain. Lachesin may thus be important in neuronal differentiation while Delta, big brain and perhaps Notch, are necessary for a cell to adopt an epidermal fate in insects. Consistent with this, Lachesin is expressed by cells in the ventral ectoderm at positions where neuroblasts first form. Whether the earliest Lachesin-expressing cells are future neuroblasts is difficult to determine without labelling the cells directly and following their development. In the limb, however, Lachesin-expressing cells delaminate from the limb epithelium, move into the limb and differentiate as sensory neurons. Thus, in both the PNS and CNS, Lachesin is expressed only on those cells that are potentially neurogenic. Doe and Goodman (1985b) showed that cells in the neurogenic ectoderm can replace an ablated neuroblast up until the time they begin to differentiate as non-neuronal cells. These ventral cells may express Lachesin throughout the period when they are competent to replace a neuroblast and lose Lachesin expression as they differentiate along non-neuronal lines.

After neurons differentiate, they extend axons that form the axonal scaffold in the CNS and segmental and intersegmental nerves in the PNS. Lachesin is expressed on growth cones and axons from the beginning of axonogenesis, then becomes restricted to a subset of axons in the CNS after the major nerve pathways have been established. Lachesin expression is thus consistent with a role in axon outgrowth. The fact that Lachesin is a GPI-linked protein expressed on the pioneer growth cones and on the guidepost cells in the limb also suggests a role in pathfinding by the limb pioneer axons. Chang et al. (1992) disrupted axonal pathfinding in grasshopper limbs by treating embryos with PI-PLC. Pioneer axons are frequently unable to navigate correctly to the CNS when GPI-linked proteins are stripped from the pioneer neurons and the embryonic environment through which they grow. Culturing experiments with anti-Lachesin antibodies and/or the Lachesin protein will allow us to test directly whether Lachesin is involved in the interactions between a pioneer growth cone and its environment that allow accurate pathfinding to the CNS.

Sequence comparisons also suggest that Lachesin plays a role in cell adhesion and axon outgrowth. Lachesin shows sequence similarity to several Ig proteins expressed in the nervous system in vertebrates and invertebrates (Fig. 9). Many of these Ig-type molecules play a role in cell recognition events and cell adhesion. Lachesin, amalgam, the poliovirus receptor and OBCAM all contain one V- and two C2-type Ig domains and thus may constitute a structural subfamily of Ig proteins (Hellen et al., 1989). The common structure of this subfamily of Ig proteins suggests both evolutionary and functional relatedness.

Drosophila Lachesin is most closely related (38% identical, 60% similar) to *Drosophila* amalgam, an Ig superfamily protein present on mesodermal cells, neurons and axons in the developing fly embryo. Amalgam can act as an adhesion molecule when artificially bound to cell membranes in vitro, but appears to be secreted by non-neuronal tissue in vivo (M. Seeger, personal communication). The next most similar proteins are all approximately 30% identical and 50-55% similar to Lachesin over the two C2-type Ig domains. These include *Drosophila* neuroglian, L1 from mouse, rat and human, chicken Ng-CAM, the human colorectal tumor suppressor protein (DCC), bovine opioid-binding cell adhesion molecule (OBCAM), rat TAG-1 and NCAM. Functional studies on some of these molecules indicate roles in cell adhesion as well as axon outgrowth. L1 is present on Schwann cells and growing axons and can mediate axon outgrowth through homophilic interactions (Lemmon et al., 1989). The GPI-linked form of NCAM, NCAM-120, promotes axon outgrowth of rat CNS neurons (Doherty et al., 1990). TAG-1 acts as a neurite-promoting factor and is present on subsets of growing axons in the rat spinal cord (Furley et al., 1990; Dodd et al., 1988).

The similarity of *Lachesin* to two human genes, the colorectal tumor suppressor (DCC) and poliovirus receptor (PVR) is intriguing. The DCC protein is a putative cell surface protein found to be absent in some colorectal tumors

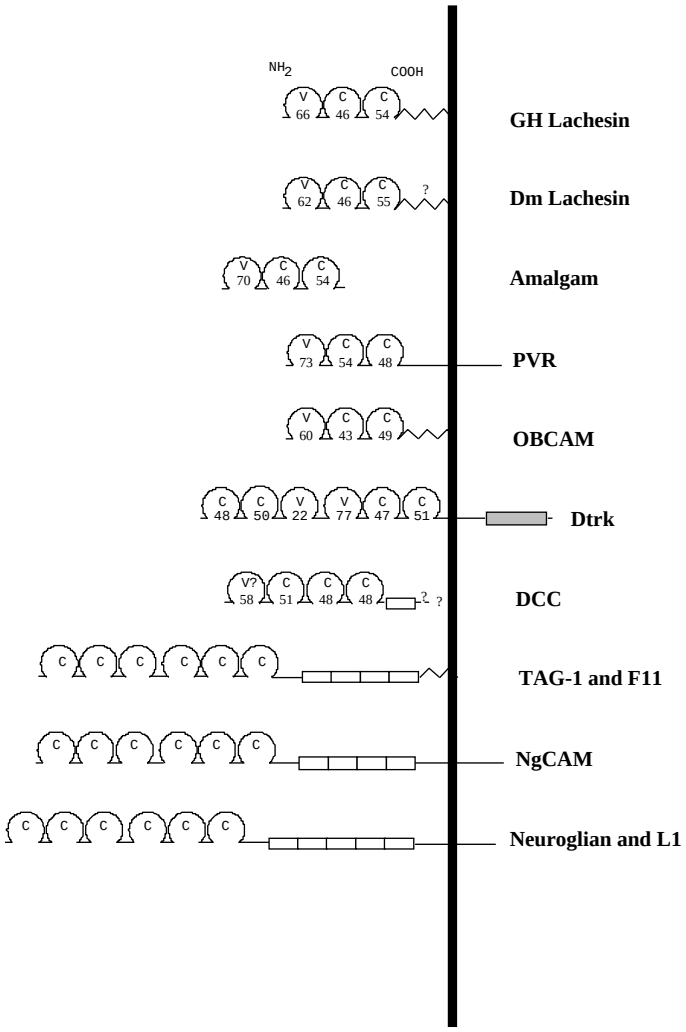


Fig. 9. Diagram of Lachesin and other related Ig superfamily proteins. Immunoglobulin domains are shown as loops. V-labelled loops are similar to variable type domains based on the presence of specific amino acids within the loop, C-labelled loops are similar to C2 type domains. The number of amino acids between the conserved cysteine residues is indicated for the smaller proteins. Open boxes represent fibronectin type III domains, hatched box represents a tyrosine kinase domain. Jagged line represents glycosyl phosphatidyl inositol attachment to cell membrane. PVR, poliovirus receptor; OBCAM, bovine opioid binding cell adhesion molecule; Dtrk *Drosophila* receptor tyrosine kinase: DCC, human colorectal tumor suppressor protein.

and has been hypothesized to act as a tumor suppressor (Fearon et al., 1990). The normal function of the human poliovirus receptor is unknown, but transfection of mouse cells with PVR cDNA is sufficient to allow viral binding and infection (Mendelsohn et al., 1989). The existence of an insect picornavirus and a cellular binding assay using cricket cells may allow a more detailed analysis of the mechanisms of virus binding (E. Wimmer, personal communication). Since Lachesin is expressed in cricket embryos in a pattern similar to that seen in grasshopper (not shown), we can directly test whether Lachesin serves as a viral receptor using anti-Lachesin antibodies.

Having identified a novel Ig protein in both grasshopper and *Drosophila*, we are now in a position to perform a number of tests of molecular function. The grasshopper nervous system provides an ideal system for experimental treatments that block Lachesin function. *Drosophila* genetics will allow us to examine embryonic development in the absence of the Lachesin protein. By combining these two systems, we may learn how Lachesin contributes to the formation of the invertebrate nervous system. This in turn may shed light on more general developmental processes in both vertebrates and invertebrates.

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