Molecular gradients along the proximal-distal axis of embryonic insect legs: possible guidance cues of pioneer axon growth

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Summary

It has been proposed that gradients of environmental cues direct the proximal growth of pioneer axons in embryonic insect legs. Hybridoma techniques have been used to produce 3 monoclonal antibodies (mAbs) that bind to components associated with the basal lamina/extracellular matrix that are non-uniformly distributed along the proximal-distal axis of cockroach legs at the time of pioneer axon growth. Two of these mAbs, PROD-1 and PROD-2, label the proximal parts of the leg more intensely than the distal ends. The other mAb, DIP-1, has the reverse pattern of binding with the distal parts of the leg labeled more intensely. The graded distribution of these antigens only occurs just prior to and during the growth period of the Ti1 pioneer axons. Western blot analyses and immunoprecipitations have identified the protein antigens recognized by these mAbs. The spatial and temporal distributions of these molecules in the legs and the CNS make them good candidates for environmental guidance cues of pioneer axon growth.

Key words: molecular gradients, positional information, axon guidance, axon growth, basal lamina, cockroach.

Introduction

The growth of pioneer axons is guided by molecular cues in the environment that may be diffusible, bound to cell surfaces or bound to basal lamina/extracellular matrix (ECM). The relatively simple pioneer axons in the embryonic insect leg are well characterized in terms of the paths of axon growth (Bentley and Keshishian, 1982; Ho and Goodman, 1982; Keshishian and Bentley, 1983a,b) and the identification and location of environmental cues (Bentley and Caudy, 1983; Caudy and Bentley, 1986a,b,1987; Lefcort and Bentley, 1987; Condic and Bentley, 1989a,b,c). Experimental manipulation of the grasshopper leg has revealed that neither the underlying mesoderm nor diffusible molecules from the CNS target provide essential guidance cues for the Ti1 axon growth. Two of these mAbs, PROD-1 and PROD-2, label the proximal parts of the leg more intensely than the distal ends. The other mAb, DIP-1, has the reverse pattern of binding with the distal parts of the leg labeled more intensely. The graded distribution of these antigens only occurs just prior to and during the growth period of the Ti1 pioneer axons. However, the subsequent observation that the retracted axons can regrow proximally in the absence of the basal lamina indicates that a similar if not identical set of cues exists in the epithelial cells (Condic and Bentley, 1989c). The basal lamina and/or the basal surface of the ectodermal epithelial cells contain adhesive components that may be distributed in a graded manner along the proximal-distal axis. This gradient may be one of the many cues that guide the growth of the pioneer axons.

Several cell surface adhesion molecules have been identified in insects. Some of these have been identified by their amino acid sequence to be homologs of known vertebrate adhesion molecules (Bogaert et al., 1987; Leptin et al., 1987; MacKrell et al., 1988; Seeger et al., 1988; Klämbt et al., 1989). Others have been directly demonstrated to mediate cell-cell adhesion (Snow et al., 1989; Barthalay et al., 1990; Elkins et al., 1990; Fehon et al., 1990; Krantz et al., 1990). However, no graded distribution of any of these cell adhesion molecules has been observed in any part of the insect.

Several of the molecular components in the insect ECM are homologous to those found in vertebrate ECM (reviewed by Fessler and Fessler, 1989). There is considerable evidence from vertebrates that some of these molecules generate restricted yet permissive routes for axon growth. Some of them have been localized to the pathways along which axons grow during development in vivo.
These gradients are transient but are present during the time there is no evidence that any of them can guide this growth (Denburg et al., 1989). Mice were immunized with nerve of pioneer axon growth. The correlative results presented in any particular direction along these trails. The one attempt to orient growth cones to a gradient of laminin was not successful (McKenna and Raper, 1988). This demonstrates the need for some as yet undiscovered components of the ECM whose distribution in a spatial gradient along a permissive route can guide the direction of axon growth.

We have used hybridoma techniques in an attempt to obtain monoclonal antibodies (mAbs) that label potential cues guiding the growth of pioneer axons in the embryonic leg of the cockroach, Periplaneta americana. Three mAbs have been produced that label molecules associated with the ECM and the basal lamina and that are distributed in a graded manner along the proximal-distal axis of the leg. These gradients are transient but are present during the time of pioneer axon growth. The correlative results presented here are consistent with a role for these antigens in the guidance of the proximal growth of these axons.

Materials and methods

Collection and staging of embryos
Egg cases from laboratory colonies of the cockroach, Periplaneta americana were collected daily. They were maintained in a humidified incubator at 30°C with a 12:12 hour light:dark cycle. Under these conditions it takes 30 days for nymphs to emerge. The system of division of embryonic development into morphologically defined stages of Lenoir-Rousseaux and Lender (1970) was used. Using the average age of the embryos at which each stage is observed, they were converted to percentage of development. Further details on the precision of the staging of the embryos have previously been published (Denburg and Norbeck, 1989; Norbeck and Denburg, 1991).

Production of monoclonal antibodies
The details of the fusions yielding the hybridoma cell lines that produce the mAbs used in this study have previously been published (Denburg et al., 1989). Mice were immunized with nerve cords from stage 23-25 (43-50% development) embryos. Although the original purpose of this fusion was to obtain mAbs that bind to developmental stage specific neuronal antigens, an additional 12 hybridomas were saved because they produce mAbs which appear to bind to the basal lamina or the extracellular matrix.

Binding of monoclonal antibodies to whole mounts of embryos
The procedures for determining the pattern of mAb binding to whole mounts of embryos are essentially identical to those previously described (Denburg and Norbeck, 1989). Embryos were removed from the egg cases in the presence of fixative (4% paraformaldehyde in 100 mM phosphate buffer, pH 7.2). They were fixed for 30 minutes at room temperature and then thoroughly washed in phosphate-buffered saline (20 mM potassium phosphate, pH 7.2 and 150 mM NaCl). Embryos were incubated for 1 hour at room temperature in blocking solution (Tris-buffered saline (TBS) of 50 mM Tris, pH 7.2 and 350 mM NaCl containing 2% Triton X-100, 2% goat serum and 30 mg/ml bovine serum albumin) to prevent non-specific binding of mAb. Treatment with hybridoma supernatant, diluted 1:100 with blocking solution, was done overnight at 4°C. Unbound mAb was removed by extensive washing in TBS containing 0.2% Triton X-100. Bound MAb is detected with horseradish-peroxidase-conjugated goat anti-mouse antibodies (BioRad) diluted 1:200 in blocking solution. After an overnight incubation, the unbound secondary antibodies were removed by washing in TBS containing 0.2% Triton X-100. Treatment with diaminobenzidine (0.5 mg/ml) and H₂O₂ (0.01%) in 100 mM Tris, pH 7.2 made the MAb binding visible. The embryos were cleared in 95% glycerol and mounted for examination under the light microscope.

In some experiments the embryos were fixed in ethylene glycol bissuccinimide (EGS). This fixative was dissolved in DMSO to make a 100 mM stock solution. The working solution is a 1:10 dilution in PBS containing 0.01% Triton X-100 in which the embryos were incubated for 24 hours at 30°C. With shorter times of fixation the antigens are not detected in the ECM, presumably due to a lack of immobilization. These conditions represent the highest workable concentration of fixative and the optimal time of incubation for preservation of antigenicity. After removal of the fixative the determination of the pattern of mAb binding to the samples was performed in a manner identical to that just described. In other experiments the binding of mAbs to living embryos was determined in a manner identical to that previously described (Denburg et al., 1989).

Solubilization, western blot analysis and immunoaffinity purification of antigens
Embryos at 23% development were removed from egg cases, separated from the yolk and sonicated in PBS containing 2 mM phenylmethylsulfonyl fluoride, 50 mM EGTA, 17.5 µM leupeptin, and 5 µg/ml aprotinin to inhibit proteolytic degradation of the antigens. The sonicate was centrifuged for 1 hour at 100,000 g in an airfuge (Beckman). The supernatant was saved for analysis and the pellet was sequentially resuspended, extracted and recen trifuged in solutions of the sonication buffer to which 1 M NaCl, 2 M NaCl, 4 M urea, 1% Triton X-100 or SDS sample buffer have been added. With the exception of the final SDS extract, each of the supernatants is diluted in 4× sample buffer containing 4% SDS, 40% glycerol, 0.02 mg/ml Bromphenol Blue, 250 mM Tris, pH 6.8.

Fractionation of proteins by SDS polyacrylamide gel electrophoresis was performed by the procedure of Laemmli (1970) using 10% polyacrylamide gels for the PROD-1 antigens and 7.5% gels for the PROD-2 and DIP-1 antigens. After electrophoresis the proteins were transferred to nitrocellulose using the procedure described by Burnette (1981). Non-specific binding of mAb to the blot was blocked by incubating for 1 hour at room temperature in TBS containing 0.05% Tween and 1% non-fat dry milk. The paper was then incubated with hybridoma supernatant diluted 1:5 in blocking solution for 1 hour at room temperature. After 3 washes in TBS (5 minutes/wash), specific mAb binding was detected using horseradish-peroxidase-conjugated goat anti-mouse antisera (BioRad) diluted 1:1000 in blocking solution. The samples were incubated with secondary antibodies for 1 hour at room temperature and then washed 3 times in TBS (5 minutes/wash). The mAb binding was then visualized with the chromatic substrates diaminobenzidine (0.5 mg/ml) and H₂O₂ (0.10%) in 100 mM Tris, pH 7.2.

The immunoaffinity purification of the antigens was carried out with mAbs covalently coupled to Affi-Gel 10 (BioRad) by pro-
Molecular gradient cues for pioneer axon growth

The growth of pioneer axons in the cockroach leg

The growth of pioneer axons in the legs of cockroach embryos were observed by labeling with a neuron-specific mAb, 16-7G12, (Denburg et al., 1986). This mAb binds to a 200×10^3 M_r cytoplasmic protein present in all neurons, which appears early in development and is present while the axons are growing. The immunological label, anti-HRP, is not suitable in the cockroach. It does not label the neurons intensely and it also binds to ectodermal epithelial cells.

The first 16-7G12 labeled neurons to appear in the leg do so in the tibia and their axons follow a stereotypical proximal route with 2 changes of direction before reaching the CNS. These are the cockroach homologs of the well-characterized grasshopper Ti1 pioneer neurons (Bate, 1976; Keshishian, 1980). They pioneer the nerve branch called N5B1 (Fig. 1A). Neurons pioneering other pathways in the leg subsequently appear at stereotyped positions in the leg (Fig. 1B). They are named according to the leg segment in which they originate and the order in which they appear (see legend to Fig. 1). There is great similarity in the position and the sequence in which these neurons appear in the cockroach and the grasshopper. This is apparent by comparing Fig. 1A,B,C with Figs 4 and 6 in Keshishian and Bentley (1983b). A more detailed description of the growth of the Ti1 axons in the cockroach will be published elsewhere.

However, there remain several differences in the development of these peripheral nerves in these two insects. These include: (1) In the cockroach the growth of these pioneers occurs relatively early in development. The Ti1 pioneer neurons emerge from the epithelial layer at 18% development and most of the pathways are established by 31% development. In contrast, in the grasshopper these events start at 30% development and are completed by 44% development. (2) Considerable attention has been directed in grasshoppers to the circumferential, ventral turn of the Ti1 axons at the segment border between the coxa (Cx) and the trochanter (Tr) (Caudy and Bentley, 1987; O’Connor et al., 1990). In the cockroach these axons make a similar turn but within the Tr, not at any segment border. This was confirmed using mAb DSS-8, which selectively labels epithelial cells that will invaginate to form the segment border (Norbeck and Denburg, 1990; 1991). This change in direction of the Ti1 axons occurs immediately proximal to the DSS-8-labeled epithelial cells at the trochanter/femur border (data not shown). (3) In the grasshopper the Cx1 neurons are thought to provide important cues for the second change in direction of the Ti1 axons; the proximal turn from the circumferential growth (Bentley and Caudy, 1983). In the cockroach no cells are observed to be labeled with 16-7G12 in the corresponding position of the Cx1 neurons at the time of the proximal turn of the Ti1 axons nor later in development. (4) In the grasshopper both Ta1 and Ta2 axons grow towards and contact the cell body of the Ti2 neuron as they pioneer nerve branches N5B2a and N5B2b respectively (Keshishian and Bentley, 1983b). In contrast, in the cockroach only Ta2 axons contact the Ti2 cell body while the Ta1 axons grow proximally and later contact the axons of the Ti2 neurons. (5) The grasshopper Ti2 and Ta2 pioneer neurons form a separate nerve branch, N5B3. This branch does not form in the cockroach. Instead, these neurons contribute to nerve N5B2 (Fig. 1B).

Some of these differences between the two insects may reflect changes in the relative importance of certain guidance cues, particularly those involving the Ti1 axons. However, the overall similarities suggest commonality in many guidance cues, including those directing initial pioneer axon growth in a proximal direction. Hybridoma techniques were used to identify molecules that are distributed in a gradient along the proximal-distal axis of the legs at the time of pioneer axon growth.

Antigenic gradients along the proximal-distal axis of the leg

Several mAbs that bind to the basal lamina or the extracellular matrix were obtained from fusions performed with spleens from mice immunized with nerve cords from embryos at 43-50% development. In order to identify molecules involved in guidance of leg pioneer axons, this library of mAbs was initially screened for binding to whole mounts of legs from embryos at 20% development, a time at which only the Ti1 pioneer neurons are growing axons. Some of these mAbs label only the amorphous material between cells in the mesodermal mesenchyme (Fig. 3A,B,C) and are considered to be specific for the extracellular matrix (ECM). Other mAbs that label a non-cellular membranous material associated with the basal surface of the epidermal epithelia (Fig. 3D,E,F) are considered to be specific for the basal lamina (BL). Molecules functioning as directional cues of axon growth would be expected to be distributed in a graded manner in the leg. Three mAbs were produced whose binding varied along the proximal-
distal axis of the leg (Fig. 2). Two of them bind in a pattern that is high PROximally and low DIstally and they are called PROD-1 and PROD-2. One of them binds in a pattern that is high DIstally and low PROximally and is called DIP-1.

The binding of PROD-1 and DIP-1 appears to be localized to both BL and ECM (Fig. 2). Within the inner portions of the leg these mAbs do not outline individual mesodermal cells. This indicates that the binding is not uniformly associated with the plasma membranes of these cells but is probably localized to the ECM surrounding them. The intense labeling between the mesodermal mesenchyme and the ectodermal epithelial cells is probably localized to the BL. However, at this level of resolution it cannot be determined whether there is additional binding to the basal surface of the ectodermal epithelial cells. In contrast PROD-2 binding appears to be present only in the BL and absent from the ECM.

It is possible that the apparent graded distribution of mAb binding in leg whole mounts represents a histological artifact arising from the differential mAb accessibility of tissue in various parts of the leg or an optical artifact arising from the greater thickness of tissue in the proximal portion of the leg. There are 3 observations which indicate that this is not the case. First, the gradients detected by PROD-1 and 2 run in the opposite direction to that detected by DIP-1. The 2 types of mAbs have peaks of binding at opposite ends of the leg indicating that both ends of the leg are accessible to mAbs. Second, gradients of mAb binding identical to those observed in whole mounts are observed in frozen transverse sections of the leg (Fig. 2). In these 15 µm sections antigens should be fully accessible to the MAbs. Third, several other mAbs were produced that uniformly label the BL and/or the ECM throughout the leg at this stage of development. Two of these are shown in Fig. 3. mAb ECM-1 binds to the ECM within the mesoderm (Fig.
mAb BL-1 selectively labels the BL in a uniform manner along the entire length of the leg (Fig. 3D,E,F). These mAbs apparently have full access to antigens in all regions of the leg as detected by techniques that reveal graded distributions of PROD and DIP mAbs. These results also indicate that the non-uniform distribution of PROD and DIP are not the result of the non-linear chromatic reaction of HRP-conjugated secondary antibodies used to detect mAb binding.

In all these leg whole mounts and sections in which a non-uniform distribution of mAb binding is observed, the tissue was initially fixed in paraformaldehyde. It has been reported that paraformaldehyde fixation can result in the formation and detection of a non-uniform distribution of a single conformation of an antigen which is actually uniformly distributed (Ferretti and Brockes, 1990; Rabacchi et al., 1990). In systems where this phenomenon was demonstrated, fixation with ethylene glycol bis(succinimide) (EGS), an NH₂-crosslinking reagent with a very long spacer arm, revealed the antigen to actually be present throughout the tissue. When cockroach legs are fixed in EGS the graded distributions of mAb binding are still observed (data not shown).

An additional way to control fixative induced non-uniform distributions of antigen is to determine the pattern of mAb binding in unfixed, living tissue. PROD-2 labeled unfixed legs in a pattern identical to that observed in fixed, permeabilized preparations. However, PROD-1 and DIP-1 did not bind to the living tissue, indicating that without fixation and permeabilization of the tissue, these mAbs do not have access to their epitopes.

Therefore, by immunohistochemical criteria the binding patterns of PROD-1,2 and DIP-1 represent a true graded distribution of their respective epitopes. No effort has yet been made to quantitate the levels of mAb binding and to directly visualize the shape of the antigen gradients. This will be particularly difficult in whole mounts of the leg because the thickness of the tissue varies along the proximal-distal axis. In addition, the horseradish-peroxidase catalyzed reaction used to detect mAb binding is probably not linearly related to the amount of mAb bound. However, in spite of these technical limitations it is clear that the PROD and DIP antigens are distributed in a non-uniform manner along the proximal-distal axis of the leg. Qualitative observations of mAb binding performed at various stages of development will demonstrate how the antigens become distributed in a gradient, how the distributions of the antigens change in time until the gradients disappear, and how these spatial distributions of the antigens correlate with the growth of the pioneer axons in the leg.
Spatial and temporal distribution of the binding of PROD-1 in the embryonic leg

Photographs of the mAb binding patterns at 4 times in development are presented in Figs 4 and 5. A more complete presentation of the distribution of mAb binding sites throughout development is schematically drawn in Fig. 6. Each illustration in this figure contains more information than could be conveyed in a single photograph of a single plane of focus. The intensity of mAb binding is qualitatively estimated on the basis of a visual examination of at least 30 legs labeled with each mAb. In all of these figures the extent of pioneer axon growth at the same stage of development is shown. This information is obtained by longitudinally slitting embryos along the midline and labeling one half with mAb 16-7G12 and the other with a PROD or DIP mAb. Attempts to label legs with both mAbs were not successful because the binding to the BL/ECM visually interfered with the observation of the growing axons. The variability between legs as a result of a developmental gradient along the anterior-posterior axis is always less than the differences between developmental points in Fig. 6.

From the time that the leg starts to evaginate from the body wall (14% development) until 17% development the binding of PROD-1 is evenly distributed throughout the embryonic leg (data not shown). However, by 18% development, when the Ti1 pioneer neurons have emerged from the epithelial cell layer at the distal tip of the leg, a graded distribution of PROD-1 is observed. This is seen mainly as an absence of binding in distal regions of the leg. It appears as though the cells added at the growing distal end are not associated with the antigen. As the Ti1 pioneer axons first extend proximally at 18.4% development they are growing towards an increasing concentration of PROD-1 antigen. This gradient continues to exist throughout the period of proximal growth of these axons towards the CNS. The second set of neurons to extend axons proximally, the Cx2 cells (arrow in Fig. 4), pioneer the branch of nerve 3 into the leg at 22% development. These cells are given this name, even though they are the first neurons to appear in the coxa, because they are pioneers for nerve 3 which is similar to the Cx2 neurons in the grasshopper leg. They also grow in a direction characterized by increasing concentrations of PROD-1 antigen. Shortly after these axons reach the CNS (24% development) the levels of PROD-1 binding start to decrease until by 28% development there is no detectable antigen (Fig. 5). No other pioneer axons growing proximally encounter a gradient of PROD-1 antigen.

Spatial and temporal distribution of the binding of PROD-2 in the embryonic leg

From the earliest time that PROD-2 binding can be detected in the leg (16.7% development) it is distributed in a graded manner. The proximal-distal gradient of PROD-2 antigen is present before the Ti1 neurons emerge from the epithelial cell layer. Therefore, the initial proximal axon growth of these neurons, as well as all subsequent growth towards the CNS, is toward an increasing concentration of PROD-2 antigen. The graded distribution of the PROD-2 antigen is present during the period of development in which the Cx2, Tr2 and Fe2 pioneer neurons grow axons proximally. The graded distribution of PROD-2 binding gradually becomes uniform during the interval of 26-32% development as a result of an increase in mAb binding sites in more distal portions of the leg. The newly acquired antigen appears in patches initially on the dorsal surface of the leg. Eventually the leg becomes homogeneously labeled with PROD-2. Axons of the later developing Ta1 and Ta2 pioneer neurons appear to be growing on patches of PROD-2 (data not shown).

Spatial and temporal distribution of the binding of DIP-1 in the embryonic leg

In the newly evaginated leg, DIP-1 binding is observed to be uniform throughout the leg. In the interval between 16.7-19.2% embryonic development there is an increase in the
length of the legs whose distal regions have levels of the DIP-1 antigen higher than the baseline value seen in the younger legs. The Ti1 neurons emerge and initiate proximal axon growth in the presence of uniformly distributed high levels of DIP-1 antigen. Slightly later in development there is a gradual decrease in levels of the antigen in proximal regions of the leg. The resulting distal-proximal gradient of DIP-1 binding is first observed at the time when the circumferential growth of the Ti1 axon starts (Fig. 6, 19.2% development). When these axons change the direction of their growth a second time and continue again in the proximal direction the DIP-1 gradient is much more apparent (Fig. 6, 19.6% development). Axon initiation and proximal growth by the Cx2, Tr2 and Fe2 pioneer neurons occur in the presence of a gradient of DIP-1 antigen. These axons grow toward decreasing concentrations of DIP-1 antigen. In the interval between 24 and 28% development 2 transitions occur in the pattern of binding of DIP-1. By 28% development the binding which had previously been in both BL and ECM now becomes progressively lost from the ECM. Significant amounts of antigen can be observed between ectodermal epithelial cells and on the surface of the leg (Fig. 5) which may indicate that the antigen is being secreted from the interior of the leg. At this stage of development the graded distribution of the antigen within the BL is lost although occasional patches of greater binding of mAb are present. The gradient is altered by a decrease in mAb binding in the distal regions of the leg.

Spatial distribution of the binding of PROD and DIP mAbs in the embryonic CNS

In order to determine whether PROD and DIP antigens could also be involved in guiding pioneer axon growth in the CNS, preliminary studies consisting of the observation of the patterns of mAb binding to embryos at 18.5% development were performed. At this stage two of the major axon pathways, the longitudinal connectives and the anterior...
commissures, are being pioneered within the thoracic segments (Fig. 7G). Ultrastructural examinations of homologous pioneer neurons in the grasshopper have demonstrated that these axons initially grow dorsally until they reach the basement membrane and then they turn in the appropriate direction after contacting this substratum (Bate and Grunewald, 1981; reviewed by Anderson, 1990). The dorsal basement membrane around the CNS is a thickening of the basement membrane as well as portions of the CNS extending ventrally (Fig. 7A, B). PROD-2 labels only the dorsal basement membrane (Fig. 7C, D) and DIP-1 patchily labels ventral parts of the ganglia (Fig. 7E, F). No graded distributions of mAb binding in the anterioposterior axis or in the medial-lateral direction of the ganglia could be observed. Again, there is a correlation between high concentrations of PROD-1 and PROD-2 antigens and the initial direction in which pioneer axons grow. These axons also extend away from higher concentrations of DIP-1 antigen.

Preliminary biochemical characterization of antigens distributed in a gradient along the proximal-distal axis of the leg

The preliminary biochemical characterization of the antigens consists of the determination of solubility properties and relative molecular masses. Embryos at 23% development, a stage in which all the antigens are distributed in a gradient in the legs, are used as a source of antigen.

PROD-1 reproducibly (n=10) labels proteins of 110, 77, 72 and 62×10^3 M_r in western blots after fractionation by SDS-polyacrylamide gel electrophoresis (Fig. 8A). Variable, low amounts of proteins of 55 and 25×10^3 M_r are observed in different preparations. Reduction with β-mercaptoethanol in the sample buffer destroys the ability of these proteins to be labeled by the mAb. The solubility properties of all of these forms of the antigen are identical. Approximately 20% of the antigen is present in the PBS extract. Subsequent extraction with high salt solutions solubilizes nearly all the antigen, with the 2 M NaCl solution containing the highest levels. Usually only very low amounts of antigen are obtained in subsequent extractions in 4 M urea, 1% Triton X-100 or SDS sample buffer. In 30% of the preparations approximately 20% of the PROD-1 antigen remained in the pellet after all of the extractions and could be solubilized only in SDS sample buffer. Immunoaffinity purification of the PROD-1 antigens from a biotinylated PBS extract results in the isolation of the same set of mAb binding proteins (Fig. 8C). However, additional biotinylated proteins of 34, 29 and 16×10^3 M_r are specifically purified and detected with horseradish-peroxidase-conjugated streptavidin. These proteins are not labeled by the mAb in western blots, are not eluted from immobilized control mAb and probably are complexed with the antigens. PROD-1 binds to multiple proteins that exist in complexes with other proteins which do not bind the mAb, and since they are relatively easy to solubilize are likely to be absorbed to the structural framework of the ECM/BL.

PROD-2 labels a 220×10^3 M_r protein in western blots only after solubilization in 4 M urea (Fig. 8B). Again, the presence of β-mercaptoethanol in the sample buffer destroys this protein’s antigenicity. The solubilized antigen does not bind to the immobilized mAb thus preventing its immunoaffinity purification or its immunoprecipitation. From its solubility properties and its large size the PROD-2 antigen appears to be a structural component of the BL.

DIP-1 labels no proteins in western blots (data not shown). Semi-quantitative measurements of levels of the antigen in homogenates of embryos can be made using the immunodot-blot technique (Hawkes et al., 1982). Approximately 10% of the antigen is soluble in PBS. Extraction of the pellet with solutions containing high or low salt, urea or detergents does not solubilize the antigen in a form that can be recognized by the mAb. The antigen in the PBS extract is immunoaffinity purified and 3 proteins of 218, 199 and 142×10^3 M_r are specifically isolated (Fig. 8D). It could not be determined whether DIP-1 binds to all of these proteins.
Discussion

Role of molecular gradients in axon guidance

Gradients of molecules have been suggested to play several functions in the development of the nervous system. Gradients of diffusible agents produced by target tissues have been shown to exert chemotropic effects on growing axons (Lumsden and Davies, 1983, 1986; Tessier-Lavigne et al., 1988; Heffner et al., 1990; Bolz et al., 1990). Similarly, gradients of non-diffusible molecules immobilized on cell surfaces (Bonhoeffer and Gierer, 1984; Gierer, 1987; Blair et al., 1987) or basal lamina/ECM (Nardi, 1983) have been hypothesized to guide axon growth by providing a substratum containing a gradient of affinity for the growth cone.

Several candidate cell surface guidance cues distributed in gradients have been identified. Temporal retinal axons preferentially grow on membranes of anterior tectal neurons (Bonhoeffer and Huf, 1980, 1982). A glycoprotein that can cause collapse of temporal growth cones is present at highest levels in the avoided posterior tectal membranes and presumably is distributed in a gradient along the anterior-posterior axis of the tectum (Cox et al., 1990; Stahl et al., 1990). Other identified molecular gradients in the vertebrate visual system include the JONES antigen (Constantine-Paton et al., 1986) the TOP antigen (Trisler and Collins, 1987), the high affinity laminin receptor (Rabacchi et al., 1990; aldehyde dehydrogenase (McCaffery et al., 1991) and the TRAP antigen (McCloon, 1991). A rostro-caudal gradient of a glial membrane antigen has been suggested to play a role in axon guidance (Suzue et al., 1990).

Fig. 6. Schematic representation of the complete developmental time course of the binding of the mAbs to the embryonic leg. Each horizontal bar represents the leg at the labeled stage of development. The length of each bar is proportional to the length of the leg. Scale bar is 50 µm. Bold vertical lines in the bars indicate segment borders as detected with mAb DSS-8 (Norbeck and Denburg, 1991). The density of the stippling is a measure of the relative intensity of labeling by a particular mAb. The position of neuronal cell bodies, progress of axon growth, segment borders and the distribution of mAb binding can all be compared at each stage of development. The actual geometry of the axon pathways is distorted by the bar graph format.
Prior to the results presented in this communication, no gradients of molecules associated with the BL/ECM have been observed. The mAbs described here recognize 3 different antigens distributed in a gradient along the proximal-distal axis of the embryonic cockroach leg at the time of proximal growth of the pioneer axons. Previous studies on the growth of these axons in the grasshopper leg have demonstrated that the underlying epithelial cells and the BL possess a gradient of affinity for the growth cones that increases proximally (Caudy and Bentley, 1986a; Condic and Bentley, 1989c).

Graded distributions of antigens along the proximal-distal leg axis

Considerable effort was directed towards demonstrating that the observed gradient patterns of mAb binding actually represent a graded distribution of antigens rather than artifacts of the immunohistochemical techniques. For each of the mAbs, identical patterns of binding are observed in both whole mounts and frozen sections of paraformaldehyde fixed legs. In addition, gradient patterns of mAb binding are observed for all 3 mAbs in legs fixed with EGS and for PROD-2 in unfixed legs. Therefore, proximal-distal gradients of the antigens do exist as detected immunohistochemically. However, the enzyme-linked method for detecting mAb binding is non-linear, and the varying thickness of the leg along the proximal-distal axis makes it impossible to use this data to quantitatively measure the distributions of the antigens. The use of fluorescently labeled mAbs should enable better determinations of the shapes of the gradients and observations of how the growth cones of the pioneer axons interact with them.

One explanation for the graded distribution of antigens in the BL/ECM is that it merely reflects gradients in the

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**Fig. 7.** Binding of the mAbs to the thoracic ganglion of embryos at 18.5% development. Labeling is observed primarily of dorsal structures by PROD-1 (A,B) and PROD-2 (C,D) and of ventral structures by DIP-1 (E,F). The binding is examined in serial transverse sections (A,C,E) and longitudinal sections (B,D,F) of the thoracic region of the nerve cord. In all of these sections dorsal is towards the top and in B,D,F anterior is towards the left. The state of axon growth at this stage of development is made visible in whole mount by labeling with neuron-specific mAb 167G12 (G). Pioneer axons of the anterior commissure (solid arrows) are growing towards the mid-line and those of the longitudinal connectives (open arrows) have grown to the anterior margin of the ganglion. Scale bar is 20 µm in all parts.
level of differentiation. Since the leg grows primarily from the distal end, proximal parts of the leg are developmentally more advanced than the distal parts. Perhaps the PROD-1 and -2 antigens are present at high levels proximally because they are characteristic of a more developmentally advanced state of the BL/ECM. This does not appear to be a viable explanation for the following reasons. (1) The graded distribution of PROD-1 is altered at later stages of leg development by a loss of antigen. Its presence in proximal regions of the leg does not presage a later, more differentiated state because this state is characterized by the absence of PROD-1. (2) At the time when these 3 antigens are distributed in a gradient along the proximal-distal axis of the leg, several other components of the BL/ECM are labeled uniformly throughout the leg by other mAbs. Therefore, at the stages of development examined no gradient of differentiated state of the BL/ECM is detected by the distribution of antigens like BL-1 and ECM-1. (3) The DIP-1 antigen is present at high concentrations in the later developing distal leg regions and later becomes distributed uniformly in the leg. Its presence certainly does not correlate with a mature state of the BL/ECM. In fact, it does not even correlate with an immature state of the BL/ECM. (4) The gradients of each antigen are generated in different manners, none of which follow the overall proximal-distal gradient of leg development. PROD-1 and DIP-1 are initially distributed uniformly in the leg. The gradient of PROD-1 forms because new growth in distal parts of the leg lacks the antigen. The gradient of DIP-1 forms as a result of an increase in basal levels of the antigen in distal parts and a loss of antigen from proximal parts of the leg. PROD-2 is present in a gradient from the earliest time it is detected.

Another possibility is that rather than directing axon growth, the antigen gradients are the result of axon growth. However, PROD-2 and DIP-1 gradients exist in regions of the leg prior to their receiving growing axons. The loss of PROD-1 antigen in the distal regions appears to correlate with the emergence of the Ti1 neurons and the initiation of their axon growth. However, there is an additional increase in the level of this antigen in the proximal region of the leg prior to the entrance of the pioneer axons. Therefore, the mechanisms generating the gradients of the antigens are not coincident in time or space with the growing pioneer axons.

**Functions of antigens distributed in gradients along the proximal-distal axis of the leg**

The evidence for the role of the PROD and DIP antigens as axon guidance cues is so far only correlative in nature. The spatial and temporal distributions of the antigens suggest their involvement in axon pathfinding. These antigens are localized to the BL and the ECM. In grasshoppers, ultrastructural studies have shown that the filopodia at the growth cones of the Ti1 pioneer neurons are most often in contact with the BL underlying the ectodermal epithelial cells (Anderson and Tucker, 1988). Enzymatic digestion of the BL at a time before these neurons have contacted differentiated segment borders or neurons results in the retraction of the growth cone and axon (Condic and Bentley, 1989a). However, the ability of Ti1 axons to

Fig. 8. Binding of PROD-1 (A) and PROD-2 (B) to proteins after fractionation by SDS-polyacrylamide gel electrophoresis (PAGE) and transfer to nitrocellulose. Sonicated embryos (23% development) are sequentially extracted in PBS (1) and in PBS containing 1 M NaCl (2), 2 M NaCl (3), 4 M urea (4), 1% Triton X-100 (5) and SDS sample buffer (6). All solutions additionally contain inhibitors of proteolytic enzymes. After each extraction the samples are centrifuged at 100,000 g for 1 hour, the supernatants saved and the pellets resuspended in the next solution in the sequence. Where necessary, the supernatants are diluted in SDS sample buffer and electrophoresed in gels of 10% acrylamide (PROD-1) or 7.5% acrylamide (PROD-2). (C) Immunoaffinity purification of PROD-1 antigen. A biotinylated PBS extract is first incubated with an immobilized control mAb to a chick spinal cord antigen (lanes 2, 4) and then to immobilized PROD-1 (lanes 1, 3). After extensive washing, of the immobilized mAbs, bound proteins are eluted in SDS sample buffer, fractionated by SDS-PAGE and transferred to nitrocellulose. mAb binding (lanes 1, 2) and total protein (lanes 1, 2, 3, 4) are detected. (D) Immunoaffinity purification of DIP-1 antigen. Similar procedures result in the specific elution of 3 proteins from immobilized DIP-1 (lane 1) that are not eluted from the immobilized control MAb (lane 2). Total protein is stained with HRP-streptavidin.
regrow proximally in the absence of the BL and to now be resistant to a second treatment with elastase indicates that proximal directional cues must also be present on the basal surface of the epithelial cells (Condic and Bentley, 1989c). Since epithelial cells produce many components of the BL (Ashhurst, 1985), it is possible that these cells are the source of the guidance cues localized to the cell surface and the underlying BL. The light microscope immunohistochemistry employed in this study has not enabled us to distinguish mAb binding to BL from binding at the basal surface of the epithelial cells.

The graded distributions of the antigens also correlate temporally with the growth of pioneer axons. The proximal growth of the Ti1 and Cx2 axons occurs along a substratum that contains gradients of all 3 antigens. The proximal growth of the Tr2 and Fe2 axons occurs in a gradient of PROD-2 and DIP-1. Only PROD-2 is still distributed in a gradient when the tarsal and Ti2 pioneer neurons grow axons. At the stage of pioneer axon growth when the antigens are distributed in a gradient in the cockroach leg, ultrastructural inhomogeneities in the BL are observed in the grasshopper leg (Anderson and Tucker, 1989). BL in proximal regions of the leg is thicker and has a higher concentration of 30 nm granules than that in distal regions of the leg. It is possible that some of these structural differences in the BL are the result of the graded distribution of the antigens described here.

The observation of proximal growth of Ti1 pioneer axons in isolated legs has been suggested to indicate that gradients of diffusible neurotropic factors from the CNS are not involved in guidance of these axons (Lefcort and Bentley, 1987). However, many factors, such as the fibroblast growth factors, have a high affinity for the heparan sulfate side chains of proteoglycans localized to the BL/ECM or to cell surfaces (reviewed by Klagsbrun, 1990; Ruoslahti and Piovant, 1991). The solubility properties of the PROD-1 antigens are consistent with their being secreted proteins that bind to proteoglycans.

Leg pioneer axons grow proximally along a gradient of increasing affinity for the growth cones (Caudy and Bentley, 1986a). Many of these growth cones encounter increasing concentrations of PROD antigens and decreasing concentrations of DIP antigens. It is also possible that all of these antigens are directly responsible for the gradient of affinity for the growth cone. The DIP antigens may decrease the affinity of distal regions of the BL, actively repel the growth cone or even cause its collapse (reviewed by Patterson, 1988; Keynes and Cook, 1990). The PROD antigens may increase the affinity of the proximal BL for the growth cones. It is also possible that it is the relative ratios of these substratum associated antigens distributed in different gradients in the leg that determines the affinity for the growth cones. The spatial distributions of these antigens in the CNS is also consistent with their playing analogous roles in the initial guidance of pioneer axons in the dorsal direction. Future experiments will attempt to determine more directly the role of these antigens in axon guidance. However, it is also possible that these transient gradients are markers of positional information that are important in other developmental events such as leg segmentation.

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References


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