

Expression of epithelial alkaline phosphatase in segmentally iterated bands during grasshopper limb morphogenesis

Wesley S. Chang*, Karen R. Zachow and David Bentley

Division of Neurobiology, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

*Author for correspondence

SUMMARY

Although the study of rostral-caudal segmentation of the insect body has been a rich source of information about embryonic pattern formation, relatively little is known of the process of proximal-distal segmentation of insect appendages. Here we demonstrate that during the period of limb segmentation, five segmentally iterated, sharply demarcated bands of cell surface alkaline phosphatase activity are expressed in embryonic grasshopper limbs. These bands span each intersegmental boundary in the limb as well as one boundary within the tarsus. Within appendages, expression is restricted to epithelial cells, where activity is present on both apical and basolateral surfaces. This epithelial alkaline phosphatase remains active at neutral pH, is insensitive to levamisole inhibition, and is strongly inhibited by nucleoside monophosphates. Treatment of embryos with phosphatidylinositol-specific phospholipase C releases almost all visible chromogenic activity, indicating that the epithelial alkaline phosphatase is anchored to the

plasma membrane by glycosyl-phosphatidylinositol. When material released by phosphatidylinositol-specific phospholipase C is separated on native polyacrylamide gels, a single broad band of enzymatic activity is detected following incubation with substrate. A polyclonal antiserum raised against a $55 \times 10^3 M_r$ alkaline phosphatase from shrimp recognizes a single band of $56 \times 10^3 M_r$ on immunoblots of grasshopper membrane proteins. The spatially restricted expression of epithelial alkaline phosphatase suggests that it may be involved in epithelial cell rearrangements or shape changes associated with limb segmentation and morphogenesis. It also may contribute to definition of axon routes in the limb, since pioneer afferent growth cones turn at, and migrate along, the edge of one alkaline phosphatase-expressing epithelial domain.

Key words: alkaline phosphatase, *Schistocerca americana*, limb development, epithelial morphogenesis, PI-PLC

INTRODUCTION

The insect body plan features distinctive proximal-distal segmentation of appendages as well as rostral-caudal segmentation of the body. Development of body segmentation along the anterior-posterior axis in *Drosophila* has become a model system for analysis of embryonic pattern formation (reviewed by Ingham and Martinez-Arias, 1992). During early embryogenesis in *Drosophila*, expression of genes within circumferential bands of epithelial cells progressively defines body segmentation and differentiation within body segments.

The degree to which similar processes underlie the generation of appendage segmentation is not yet known. Transplantation experiments with adult cockroach limbs have suggested that a series of similar, iterated positional fields, corresponding to major limb segments, may be established along the proximal-distal axis (Bohn, 1970, 1971; French, 1981). In *Drosophila*, genes, including *Distal-less* (Cohen et al., 1989) and *decapentaplegic* (Gelbart, 1989), have been identified which appear to contribute to the general differentiation of the distal from the proximal end of the

appendage. In cockroaches, at least three proximal-distal axis molecular gradients are present during limb differentiation (Norbeck et al., 1992). Interaction of segmentally repeated information with proximal-distal information might lead to differentiation of the distinctive characteristics of each limb segment.

If segmentally iterated positional fields are present in limbs, they might be molecularly distinguishable. General evidence for this view was provided by the observation that serum antibodies generated against cell membranes from a portion of one cockroach limb segment recognize membranes from the corresponding portion of other segments (Bulliere et al., 1982). In leg imaginal disks of *Drosophila* pupae, the pair-rule gene *hairy* is expressed in a pattern of circumferential bands (Carroll and Whyte, 1989), which may be related to segmentation. Segmentally iterated expression of a specific molecule in insect limbs was demonstrated by Norbeck and Denburg (1990), who showed that a circumferential band of epithelial cells constituting the distal portion of each segment of embryonic limbs expresses the DSS-8 antigen, a $164 \times 10^3 M_r$ transmembrane glycoprotein (Wang et al., 1992). In the

grasshopper embryo, a similar set of cells expresses annulin, an intracellular, membrane-associated protein similar to mammalian transglutaminases (Bastiani et al., 1992; Singer et al., 1992). Recently, a different set of intrasegmental bands of epithelial cells has been distinguished in embryonic grasshopper limbs by expression of fasciclin IV, a novel transmembrane glycoprotein (Kolodkin et al., 1992).

Here, we describe a new set of segmentally iterated circumferential epithelial bands in the limb buds of grasshopper embryos. Unlike the annulin and the fasciclin IV bands, these bands span intersegmental boundaries. The cells making up the bands are distinguished by expression of a glycosyl-phosphatidylinositol (GPI)-anchored epithelial alkaline phosphatase (e-AP). GPI-anchored molecules are of particular interest in grasshopper limbs because enzymatic removal of such molecules has been shown to disrupt a highly stereotyped turning decision by pioneer growth cones at a specific axial location in the limb (Chang et al., 1992). We also show that this change in growth cone behavior occurs at the border of one e-AP expressing epithelial domain.

MATERIALS AND METHODS

Embryos and materials

Embryos were obtained from a colony of *Schistocerca americana* maintained at the University of California at Berkeley. They were dissected in grasshopper saline and staged according to the percentage of embryogenesis completed based on external morphology (Bentley et al., 1979; Caudy and Bentley, 1986). Embryos were examined from the 20% stage through the 70% stage of development. Unless specified, all reagents were purchased from Sigma Chemical Co., St. Louis, MO. Highly purified recombinant phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* (Henner et al., 1988) was generously provided by Dr Martin Low (Columbia University).

Enzyme histochemistry

Detection of endogenous alkaline phosphatase (AP) activity (Wolf et al., 1967) in whole grasshopper embryos was carried out using a modification of the procedure reported by Zackson and Steinberg (1988). Embryos were dissected out of their egg cases into saline and their dorsal closures were opened to ensure access of reagents to the inner lumen. They were fixed in 3.7% formaldehyde in AP buffer (APB; 0.1 M Hepes, 5 mM MgCl₂, 1 mM CaCl₂, pH 7.4) for 30 minutes with agitation. Embryos were then rinsed in three changes of APB over 30 minutes. To detect intracellular as well as cell surface enzyme activity, embryos were incubated for 10-20 minutes in APB containing 0.1% saponin, then rinsed again in APB before being placed in substrate solution.

Two chromogenic substrate systems were employed to visualize AP activity: 175 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP or X-phosphate, Boehringer-Mannheim) and 350 µg/ml nitro blue tetrazolium (NBT, Boehringer-Mannheim) in 0.1 M diethanolamine, 5 mM MgCl₂, pH 9.5 yielded an intense purple reaction product. Alternatively, the AP substrate kit III (Vector) was used according to manufacturer's directions to produce a turquoise blue reaction product. Embryos were incubated in either AP substrate solution for 20-40 minutes at room temperature with agitation in the dark. The reaction was stopped by several quick washes with APB, sometimes containing 10 mM ethylenediamine

Table 1. Inhibitors of e-AP activity

Compound	Concentration (mM)	Degree of inhibition
levamisole	1.0	-
	10.0	-
tetramisole	1.0	-
	2.0	-
L-phenylalanine	1.0	-
	5.0	-
EDTA	10.0	+++
sodium orthovanadate	1.0	++
	5.0	+++
ATP	1.0	-
	5.0	+
	10.0	++
AMP	1.0	++
	5.0	+++
	10.0	++++
adenosine	1.0	-
	10.0	-
IMP	1.0	++
	5.0	+++
	10.0	++++
inosine	1.0	-
	10.0	-

Inhibition studies were performed on 30%-45% stage embryos. The compounds were added to post-fixation wash buffers and the AP substrate buffer at the indicated concentrations. Embryos were developed under identical conditions (i.e. time, temperature) within each experiment ($n=12$), and were visually assayed for inhibition of AP reaction product deposition by comparison with untreated control embryos, as well as embryos treated under other conditions. The degree of inhibition is scaled as follows: -, no inhibition (same as control); +, just detectable inhibition of AP activity; ++, approximately 50% inhibition compared to control; +++, nearly complete inhibition; +++++, complete inhibition (no detectable AP activity).

tetra-acetic acid (EDTA). Background staining was reduced by incubation of embryos in 100% methanol for 3-5 minutes with agitation. Embryos were then rehydrated in phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM NaCl, pH 7.4) and taken through increasing concentrations (50, 70, 90%) of glycerol in PBS for whole-mount microscopy, or processed for double labeling with antibodies. Some limbs were dehydrated in ethanol, placed in propylene oxide, then embedded in Epon/Araldite and sectioned at 3-6 µm on a Sorvall microtome.

To test for inhibition of AP activity, various compounds, listed in Table 1, were added to the wash and substrate buffers during the staining procedure. Visual estimation of reduction in the intensity of staining, compared with control, was used as a semi-quantitative indicator of AP inhibition.

Double labeling studies

To visualize the developing nervous system, embryos stained for AP activity were double labeled with antibodies to horseradish peroxidase (anti-HRP), which recognize grasshopper neurons (Jan and Jan, 1982; Snow et al., 1987). Embryos were rinsed in PBS/0.5% bovine serum albumin (BSA)/0.1% saponin (PBSS) for at least 30 minutes following AP enzyme histochemistry. Rabbit anti-HRP (Cappel) was applied at a dilution of 1:5000 in PBSS overnight at 4°C. After extensive washing in PBSS, a biotin-avidin-HRP intensification kit (Vector) was used according to manufacturer's directions. The HRP reaction product was visualized using 0.5 mg/ml diaminobenzidine and 0.015% H₂O₂.

Membrane preparation, PI-PLC treatment and AP activity PAGE

Preparations of embryonic grasshopper membrane proteins were made using a modification of the protocol of Patel et al. (1987) for isolation of membranes from *Drosophila* embryos. Briefly, grasshopper eggs staged between 35% and 40% of embryonic development were dechorionated in 50% bleach, rinsed, then passed through a strainer. The collected embryos were homogenized in 10 ml of ice-cold hypotonic medium [10 mM Tris, pH 8.0, 1 mM EDTA, 20 µg/ml phenyl-N-methyl sulfonic acid (PMSF), and 1 µg/ml each pepstatin A, leupeptin, antipain, aprotinin, N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and sodium-p-tosyl-L-lysine chloromethyl ketone (TLCK)], and spun for 10 minutes at 4000 g. The supernatant was saved, and the pellet was rehomogenized and spun again. The pooled supernatants were then layered onto a cushion of hypotonic medium containing 0.25 M sucrose and spun for 1 hour at 100,000 g. The membrane pellet was resuspended in PBS containing 20 µg/ml PMSF.

AP enzymatic activity could be detected in the membrane preparations using a dot-blot assay, as well as non-denaturing gel electrophoresis. Membrane proteins (0.2-1.0 mg) were diluted to 20 mg/ml in PBS containing 20 µg/ml PMSF, then incubated with 5 Units/ml of PI-PLC (one Unit is defined as ability to hydrolyze 1 µmol phosphatidylinositol/minute), or an equal volume of carrier (50% glycerol in 50 mM Tris, pH 7.4) as control, for 1 hour at 30°C. The tubes were then spun at 100,000 g for 1 hour. Control and PI-PLC-treated pellet and supernatant samples (2-10 µl) were dot-blotted onto nitrocellulose, or run on 5% polyacrylamide minigels under non-reducing, non-denaturing conditions. The native gels were run at 10 mA constant current at 4°C using a Tris-HCl buffer system (Laemmli, 1970). The nitrocellulose membranes and native gels were then assayed for AP activity by incubation with BCIP/NBT substrate in 0.1 M Tris, 5 mM MgCl₂, 1 mM CaCl₂, pH 9.5.

Immunoblot of grasshopper membrane proteins

Murine polyclonal antibodies were generated against shrimp alkaline phosphatase (United States Biochemical Corp.). Three injections of 100 µg of protein were administered intraperitoneally, with intervals of three weeks separating the boosts. On silver stained gels, the shrimp alkaline phosphatase antigen appeared as a single band at approximately $55 \times 10^3 M_r$; antiserum taken 8 weeks after the initial injections recognized this band on an immunoblot. This antiserum was used to probe immunoblots containing embryonic grasshopper membrane proteins.

Membranes were prepared from 35%-45% stage grasshopper embryos dissected from eggs and stripped of all yolk cells. 125 embryos were homogenized in 0.3 ml of hypotonic buffer containing protease inhibitors (see above), and then centrifuged at 4000 g for 10 minutes at 4°C. The supernatant was saved and the pellet was rehomogenized in hypotonic buffer and centrifuged as before. Pooled supernatants were centrifuged at 100,000 g for 1 hour at 4°C. The membrane pellet was resuspended in 0.1 ml PBS containing 0.1 mM PMSF; the protein content was typically 20 mg/ml.

Membrane protein samples were separated by SDS-PAGE on 8% polyacrylamide gels (Laemmli, 1970), and electrophoretically transferred to nitrocellulose (Burnette, 1981). Blots were blocked for 2 hours at room temperature (rt) in TBS (50 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 5% BSA, and incubated overnight with a 1:1000 dilution of the shrimp AP antiserum in blot buffer [TBS/1% BSA/0.05% Tween 20] at 4°C. Blots were washed at rt in blot buffer six times over 1 hour, and then incubated with a 1:2000 dilution of goat anti-mouse IgG conjugated to HRP in blot buffer for 2 hours at rt. After three washes in blot

buffer and three washes in TBS, blots were developed using 0.5 mg/ml diaminobenzidine and 0.015% H₂O₂ in TBS.

RESULTS

Expression in embryonic limb buds

Using insoluble chromogenic substrates, endogenous AP activity was detected in a region-specific pattern in embryos of the grasshopper *Schistocerca americana* (Fig. 1). The expression of AP is associated with the progressive segmentation of the limb. Limb bud formation is initiated at about the 22% stage of development by evagination of the thoracic body wall. At this stage, AP activity is concentrated in distal, posterior regions of the epithelium of each presumptive limb (Fig. 2A,B). At the 30% stage, when the limb bud is elongating, but before any external signs of limb segmentation are evident, AP activity comprises a single broad band that covers much of the distal half of the limb bud. At the 33.5% stage, three separate bands are distinguishable (Fig. 2C,D): a narrow band occurs at the limb tip within the prospective tarsus, a broad band spans much of the prospective tibia and the distal region of the femur, and a very narrow band about three epithelial cells in width spans the femur-trochanter segment boundary (this boundary is marked by a pronounced inflection in the curvature of the epithelium; Caudy and Bentley, 1987). Shortly thereafter, the tibia-femur band separates into two distinct bands which span the tarsus-tibia, and tibia-femur segment boundaries. In the period of 35%-40% of embryogenesis, when the limb segments become clearly morphologically differentiated, five separate circumferential bands of AP activity are present (Fig. 2E,F): one band within the tarsus, and one spanning each of the tarsus-tibia, tibia-femur, femur-trochanter and trochanter-coxa segment boundaries. The band of staining at the trochanter-coxa boundary is both the weakest and last to arise. During this period, the width of the bands is roughly proportional to the length of the segments they occupy. Strong AP activity is also evident along epithelial cells invaginating and extending during the formation of apodemes, including the retractor unguis apodeme (Fig. 2E, arrow) and the extensor tibia apodeme.

In embryos older than the 45% stage, AP activity is greatly reduced in proximal limb segments (Fig. 2G,H). By the 50% stage, staining in the limbs becomes limited to a small band at the tip of the tarsus, a broad band in the distal part of the tibia and proximal tarsus, small patches in the proximal tibia and distal femur, and weak bands at the intratarsus, femur-trochanter and trochanter-coxa boundaries (Fig. 2G,H).

AP activity in other regions

The presence of AP activity was histochemically revealed in circumferential bands in all other appendages, including antennae, mouthparts, and terminal cerci (Fig. 1). Staining in antennae was similar in intensity to that in limbs, while activity in mouth parts appeared much weaker. As in the developing limb buds, AP activity was first detected as a broad band in the distal regions of antennae and mouth parts. Later in development, as these appendages elongate

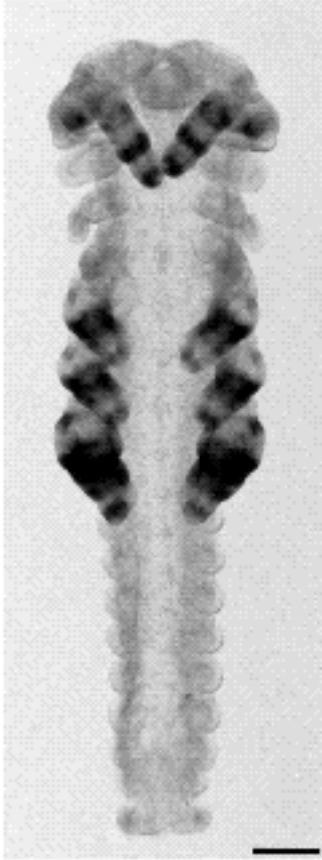


Fig. 1. Expression of endogenous AP activity in a grasshopper embryo at the 35% stage of embryogenesis. Dense staining of the ectodermal epithelium in limbs and antennae is present in a pattern of circumferential bands. Lower levels of enzyme activity are visible in lateral regions of the head, including the developing eye, and in the cerci. Scale bar, 250 μ m.

and become segmented, the single band of activity segregates to form discrete bands, and independent bands arise at other locations along the appendage. Weak AP activity also was present in the pleuropodia, in the (rudimentary) abdominal appendage buds, and in the cerci.

Strong AP activity was consistently detected within the head of embryos older than the 30% stage, particularly in a layer of sub-retinal cells within the developing eye. These AP-positive cells did not appear to co-label with anti-HRP antibodies, suggesting that they were not differentiated neurons. Upon overnight incubation with substrate, many cells within the brain, especially in the antennal lobes, of embryos older than 35% labeled for AP activity; many neurons in the adult cockroach brain express AP activity (Verhaert et al., 1990). Cells within the ventral CNS, especially near the midline of each thoracic and abdominal segment also were positive for AP activity, although the level of activity was considerably lower than in the appendages. In older embryos, each segmental ganglion contained several more laterally located cells, which were also weakly AP-positive.

Cell types expressing AP

Limbs stained for AP activity were sectioned to examine

the cellular and subcellular distribution of the enzymatic reaction product. Fig. 3 shows tangential, longitudinal, and cross sections through the tibia-femur band of labeling. The cell bodies of the two T11 pioneer afferent neurons, as well as many mesodermal cells, are found at this location (Fig. 3B,C, large arrows). All staining was confined to the epithelium; no mesodermal cells or afferent nerve cells within the limb were labeled with AP reaction product (Fig. 3B,C, arrows). Because the activity is expressed predominantly by epithelial cells, we will refer to it as epithelial alkaline phosphatase, or e-AP.

At most locations, the edges of circumferential bands of e-AP expressing epithelial cells were quite straight (Figs 2E, 3A). The narrowest bands, those at the femur-trochanter (Fig. 2D) and trochanter-coxa (Fig. 2E), were only 2-3 epithelial cell diameters in width. Transitions from labeling to non-labeling epithelium were abrupt (Fig. 3A,B), with few cells evincing intermediate levels of activity (however, the deposition of enzymatic reaction product may not precisely correspond to localization of the enzyme). At most of the developmental stages examined, the e-AP bands all extended completely around the limb circumference (Fig. 3C). The AP reaction product was distributed asymmetrically around the limb circumference, with more reaction product accumulating along the dorsal and anterior sectors of the limb epithelium.

The limb epithelium has a stratified appearance because of the packing of the nuclei, but each cell has an apical and a basal end-foot. Strong labeling was evident throughout the thickness of the epithelium (Fig. 3B,C). This suggests that, although the presence of a GPI-anchor seems to target proteins to the apical membrane compartment in mammalian polarized epithelial cell lines (Lisanti et al., 1989), the e-AP activity is strongly represented in both apical and basolateral compartments. Staining was confined to the cell perimeter region, which could reflect labeling of the plasma membrane, the thin layer of cytoplasm surrounding the nucleus, or the extracellular matrix. Labeling did not appear to be associated with the basal lamina. *In vivo* treatment with 0.02% ficin or elastase, which has been shown to digest and remove the basal lamina in intact grasshopper limbs (Condic and Bentley, 1989a), had no effect on the distribution or intensity of AP staining.

Cell-surface localization of e-AP activity

A variety of AP isoforms can exist as membrane-bound cell surface ectoenzymes or as secreted products (McComb et al., 1979). Several lines of evidence demonstrate that the e-AP activity observed in grasshopper limb buds is primarily localized to the cell surface. When embryos were lightly fixed and not permeabilized, normal patterns of staining were seen in all appendages. Fig. 4A shows a mesothoracic limb from a 34% stage embryo that was fixed for 30 minutes in 3.7% formaldehyde and then stained for e-AP activity without exposure to saponin or other detergents. The resultant labeling pattern is very similar to that in permeabilized limbs at this stage (Fig. 2D). In addition, embryos were labeled for e-AP activity *in vivo* by placing them for 30-60 minutes into grasshopper saline that contained AP substrate reagents. The intensity of staining observed was much weaker than that of lightly fixed

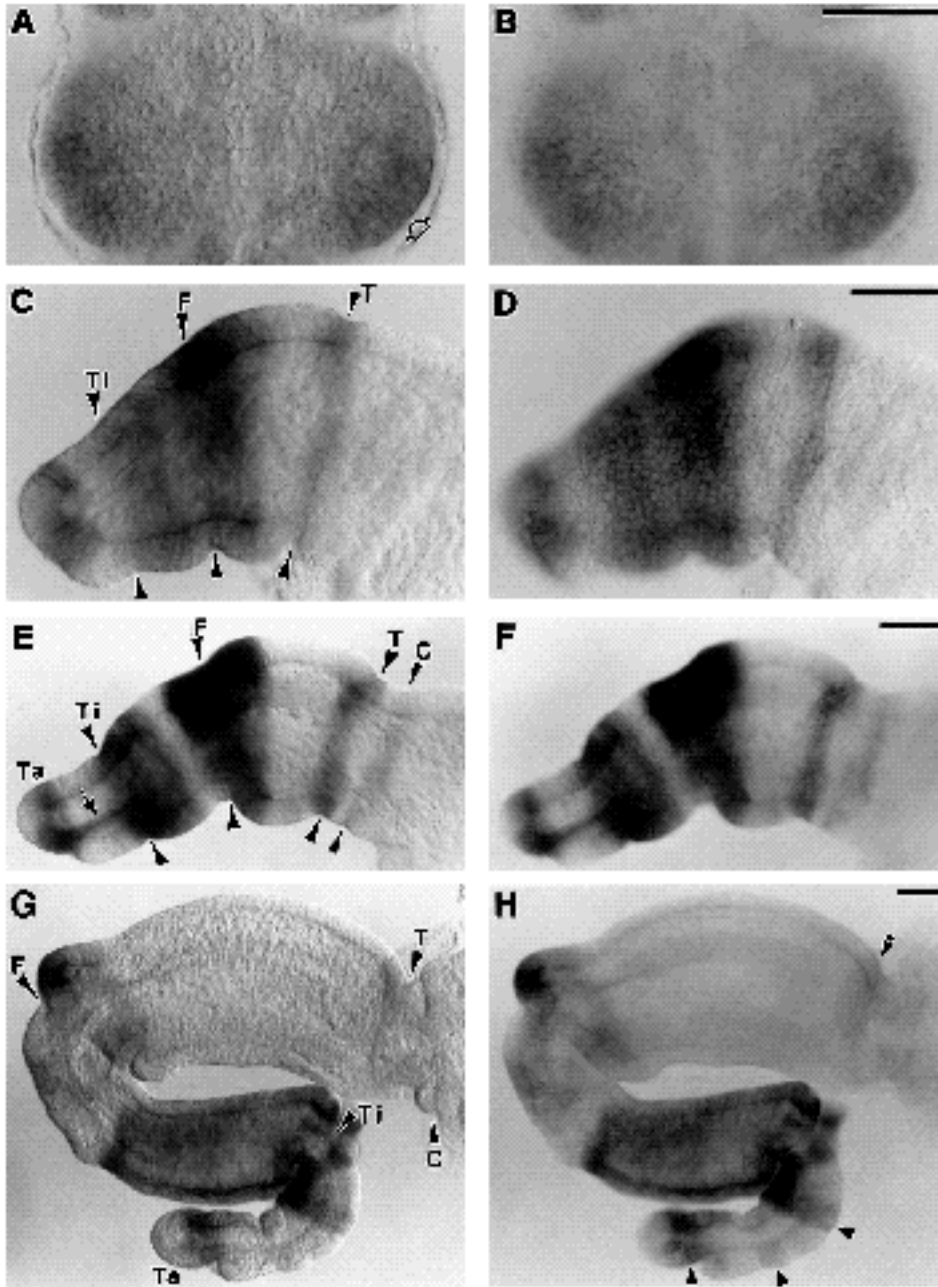


Fig. 2. Time course of expression of AP activity in metathoracic (T3) limb buds from 22-50% stage embryos. A, C, E and G were photographed at an equatorial plane of focus with DIC optics to show the outline of the limb, including developing segment boundaries. B, D, F and H show the same limbs photographed at a superficial focal plane to show cellular detail of AP expression in the epithelium. (A,B) Dorsal view of the metathoracic segment at the 22% stage, showing AP activity concentrated in distal and posterior regions of the early limb buds (arrow, A). (C,D) At the 33.5% stage, AP activity is present in three circumferential bands. Narrow bands are present near the tip of the tibia and at the femur-trochanter segment boundary, while a broad region of AP staining from the distal tibia to the mid-femur is beginning to segregate into two separate bands. In D, the outlines of epithelial cells that express the AP activity are apparent, as is a sharp boundary between AP-positive and AP-negative cells in the trochanter. (E,F) At the 40% stage, five discrete bands are visible: a band spans each of the four segment boundaries, and a fifth band lies within the tarsus. In E, invaginating cells at the tip of the tarsus, which will form the retractor unguis apodeme (small arrow) express AP activity. (G,H) At the 50% stage, a broad band of activity extends from the mid-tibia through the first tarsal segment. Labeling also occurs at the tip of the tarsus, and at the distal end of the femur. Weak labeling is evident at the femur-trochanter (double arrowhead) and

trochanter-coxa boundaries, and between tarsal segments (H, single arrowheads). Note the relative absence of staining in the mid-femur, which is the region of densest AP staining in limbs of younger embryos. (A,B) Anterior, up; (C-H) dorsal, up; distal, left. (C,E,G) Ta, tarsus; black arrowheads denote the distal boundaries of the following limb segments: Ti, tibia; F, femur; T, trochanter; C, coxa. Scale bars, 100 μ m.

embryos, but the overall spatial pattern of reaction product produced was similar. These results suggest that much of the enzymatic activity is located on the extracellular side of the plasma membrane.

Many AP enzymes examined to date are anchored to the plasma membrane by covalently attached glycosyl-phosphatidylinositol (GPI; Chuang, 1990; Howard et al., 1987; Low and Saliel, 1988; Low and Zilversmit, 1980). One of the defining characteristics of GPI-anchored cell surface proteins is their susceptibility to release from membranes by phosphatidylinositol-specific phospholipase C (PI-PLC),

which specifically cleaves the GPI anchor. To test the hypothesis that the grasshopper e-AP activity was due to GPI-anchored ectoenzyme(s), embryos were incubated with highly purified recombinant PI-PLC from *B. thuringiensis* for 60-90 minutes in vivo, then fixed and stained for AP enzyme histochemistry. Fig. 4B shows a metathoracic limb from a 34% stage embryo that was stained for AP activity following exposure to PI-PLC. The staining in the PI-PLC-treated limb was negligible compared to untreated or control-treated limbs at similar developmental stages (Figs 1, 2C), indicating that most of the visible chromogenic activity

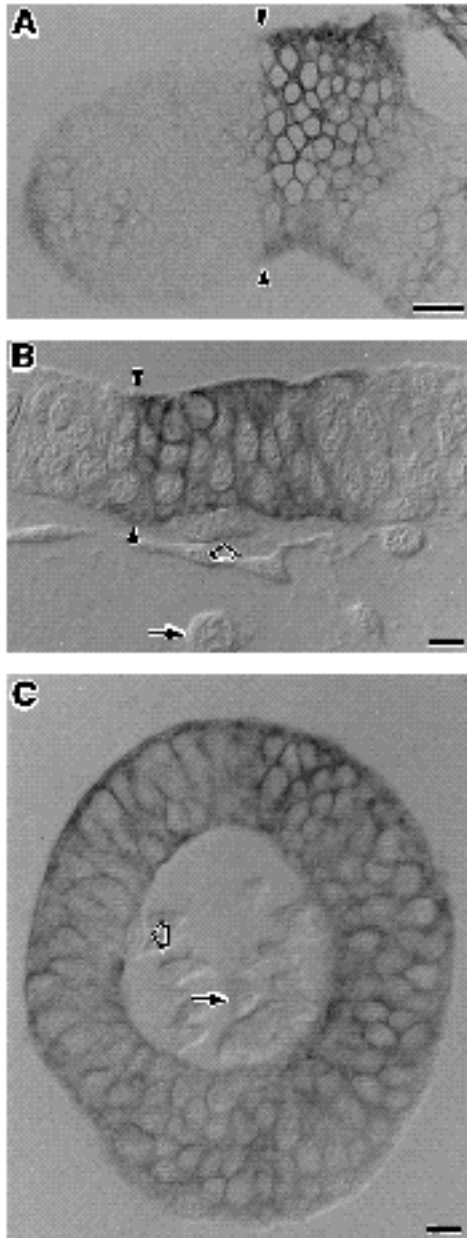


Fig. 3. Cellular distribution of AP activity in sectioned metathoracic limbs. (A) A superficial tangential section through the posterior epithelium of a 40% stage limb: the sharp boundary between AP-expressing and non-expressing epithelial cells in the mid-tibia is indicated by the black arrowheads. (B) A longitudinal section through the anterior aspect of a 40% stage limb at the tibia-femur boundary: the band of AP activity staining at the boundary is approximately 8-10 cells wide; there is a sharp border (black arrowheads) between AP-positive and AP-negative cells. Reaction product is present throughout the thickness of the epithelium, on both apical and basolateral surfaces of the epithelial cells. The Ti1 neurons (open arrow), and mesodermal cells (black arrow) do not express AP activity. (C) A cross section of a 35% stage limb at the axial level of the Ti1 neuron cell bodies: AP reaction product is present throughout the epithelial layer around the entire circumference of the limb; somewhat more activity is seen dorsally. The Ti1 neurons (open arrow) and mesodermal cells (black arrow) are not labeled. (A) Distal, left; dorsal, up. (B) Distal, left, anterior, up. (C) Dorsal, up; anterior, left. Scale bars, 10 μ m.

had been released from cell surfaces by PI-PLC. Treatment of membranes prepared from 35%-40% stage embryos with PI-PLC also caused release of AP. Activity was visualized by spotting the pellet and supernatant fractions of high-speed centrifugations onto nitrocellulose membranes; the membranes were dried, then reacted with AP substrate. Much more activity was present in the aqueous supernatant fraction of PI-PLC-treated membranes than in the supernatant of untreated membranes.

In control experiments, exposure of embryos to equivalent doses of other phospholipase enzymes, which do not cleave the GPI anchor, including phosphatidylcholine-specific phospholipase C, phospholipase B, and Type VII phospholipase D, had no effects on AP activity staining.

To distinguish further between intracellular and extracellular localization of the enzyme, embryos were treated with PI-PLC, fixed, then permeabilized with 0.1% saponin before reaction with AP substrates. Very low, yet detectable, levels of chromogenic activity were visible in the same regions of limb epithelium that stain under control conditions. In contrast, non-permeabilized, PI-PLC-treated embryos were essentially negative for AP staining. Thus, distinct cell surface and intracellular pools of e-AP enzyme activity could be visualized, with the majority of the AP activity being associated with the cell surface. The intracellular phosphatase activity could be due to either vesicle-bound enzyme being transported to, or from, the cell surface, or the activity of a different intracellular phosphatase enzyme. Taken together, these results indicate that the e-AP activity in embryonic grasshopper limbs is due primarily to one or more cell-surface enzymes that are anchored to the extracellular side of the plasma membrane of epithelial cells by GPI.

pH sensitivity of e-AP activity

To assess the pH range within which the e-AP of grasshopper embryos was enzymatically active, embryos were labeled at acidic, neutral, and alkaline pH. In most experiments, the BCIP/NBT histochemistry was performed in diethanolamine buffer at pH 9.5, while the Vector alkaline phosphatase substrate employed a Tris buffer at pH 8.2. When fixed embryos between 32-45% of development were reacted for activity using the Vector substrate at pH 7.2, the distribution and intensity of AP staining appeared similar to that at pH 8.2 (Fig. 4C). At pH 6.5, higher levels of background staining became apparent, although the overall staining pattern in the appendages was not significantly altered. At pH 6.5, a small number of mesodermal cells within the limbs and antennae, which were negative for AP activity under neutral and alkaline conditions, showed some staining; this could reflect recruitment of intracellular acid phosphatases at the lower pH. These results indicate that the embryonic grasshopper e-AP is active through a broad pH range including neutral pH values.

Inhibition of e-AP activity

Susceptibility to inhibition by different classes of compounds has been a criterion used to distinguish and categorize AP isozymes (Harris, 1989; McComb et al., 1979). Table 1 lists compounds that were tested for their ability to inhibit the e-AP activity in lightly fixed grasshopper

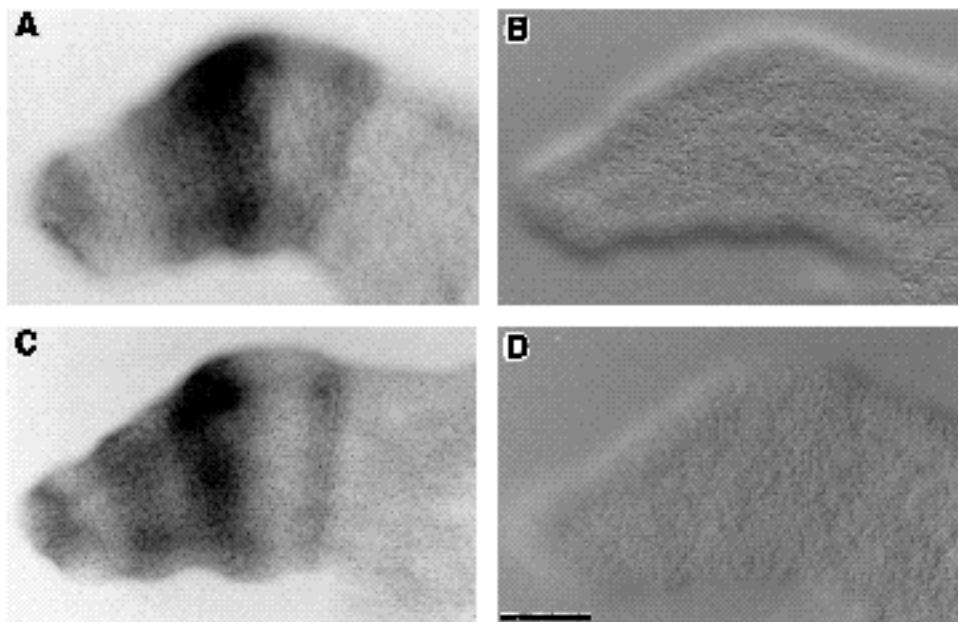


Fig. 4. Cell surface localization, pH dependence, and inhibition of AP activity. (A) AP activity in a lightly fixed, non-permeabilized limb. Strong activity persists, indicating that permeabilization with 0.1% saponin is not required for access of substrate to the AP enzyme (34% stage mesothoracic limb). (B) Incubation with PI-PLC *in vivo* releases almost all visible chromogenic activity. Thus, the activity appears to be GPI-anchored to the cell surface (34% stage metathoracic limb; the embryo was fixed immediately

after PI-PLC treatment, then processed for AP histochemistry). (C) AP activity persists at neutral pH (a 34.5% stage metathoracic limb labeled with Vector substrate at pH 7.2). (D) Incubation with 5 mM IMP almost completely inhibits visible chromogenic activity (IMP added to post-fixation rinse and substrate buffers; 34% stage metathoracic limb). (A-D) Dorsal, up; distal, left. Scale bar, 100 μ m.

embryos. Millimolar concentrations of the compounds tetramisole and levamisole (L-tetramisole) potently inhibit several non-intestinal forms of vertebrate APs in a non-competitive manner. Tetramisole and levamisole in concentrations up to 10 mM had no effect on the grasshopper e-AP activity. The amino acid L-phenylalanine is a non-competitive, stereospecific inhibitor of intestinal and some placental forms of AP, but it also did not inhibit e-AP when used in the millimolar range. Incubation with EDTA did produce substantial inhibition, suggesting that the grasshopper e-AP requires divalent cations for complete activation. However, even following exposure to 10 mM EDTA, a small amount of e-AP activity persisted in the limb epithelium.

The most effective inhibitors of the embryonic grasshopper e-AP activity were nucleoside phosphate compounds and sodium orthovanadate (Table 1). Millimolar concentrations of all phosphorylated nucleosides tested attenuated e-AP activity in a dose-dependent manner, with adenosine 5'-monophosphate (AMP) and inosine 5'-monophosphate (IMP) exhibiting the most complete inhibition (Fig. 4D). Significantly, the non-phosphorylated nucleosides adenosine and inosine did not inhibit e-AP activity up to 10 mM, while both AMP and IMP were very effective inhibitors. Both vanadate (a phosphate analog) and the nucleoside phosphates may inhibit the e-AP enzymatic activity through substrate competition. Also, the susceptibility to inhibition by nucleoside phosphate compounds suggests that the grasshopper e-AP may have intrinsic nucleotidase activity, and that extracellular nucleotides could serve as substrates for e-AP activity *in vivo*.

e-AP activity on native gels

To test the hypothesis that multiple AP enzymes could con-

tribute to the enzymatic staining in embryos and dot-blots, supernatant and pellet fractions from control and PI-PLC-treated membranes were separated on 5% polyacrylamide gels under conditions intended to optimize retention of the enzymatic activity. Upon reaction of these gels with BCIP/NBT substrate, a single, broad band of AP activity appeared in the lanes of PI-PLC-treated, but not control-treated, supernatants (Fig. 5). The band could be due to a single enzymatic species or to a small number of isozymes that migrated closely together in the gel. A strong band of AP activity at a different mobility was detected in the lanes of control membrane pellets. This band presumably represents the membrane-bound AP enzyme and may have reduced mobility either because it is normally multimerized or tightly associated with another cell-surface protein(s) prior to release by PI-PLC (Hawrylak et al., 1989), or because its association with membrane lipids inhibited its mobility through the gel matrix. In agreement with the results of *in situ* staining experiments, little AP activity was evident in the PI-PLC treated pellet.

Anti-AP serum antibody recognition of grasshopper protein

Membrane proteins isolated from dissected grasshopper embryos at 35%-45% of development were separated on an 8% polyacrylamide-SDS gel and electrophoretically transferred to nitrocellulose (Burnette, 1981). Incubation of the blot with mouse antiserum generated against shrimp AP revealed that a single band of grasshopper protein was recognized by the antiserum (Fig. 6). This grasshopper polypeptide, approximately $56 \times 10^3 M_r$, is similar in relative molecular mass to the $55 \times 10^3 M_r$ shrimp AP (Fig. 6, lane A; Chuang, 1990) and to the $56 \times 10^3 M_r$ silkworm AP (Itoh et al., 1991). Neither the shrimp AP band nor the

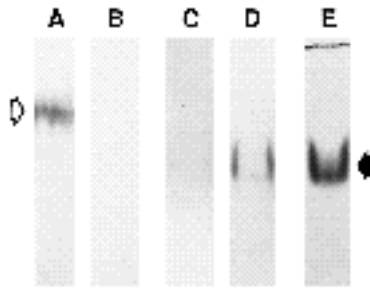


Fig. 5. AP enzymatic activity on a polyacrylamide gel run under non-reduced, non-denatured conditions. Embryonic grasshopper membrane preparations were incubated with either 5.0 Units/ml PI-PLC or an equivalent volume of carrier control. Following high-speed centrifugation, the supernatant and pellet fractions were separated on 5% gels under conditions designed to retain enzymatic activity. Gels were stained with BCIP/NBT substrate. A single, broad band of AP activity was detected in the lanes of control pellet (A, white arrow) and PI-PLC supernatant (D, E, black arrow), but not in the control supernatant (B), or PI-PLC pellet (C). Lanes A-D were run as a set using frozen membranes; lane E is from a separate experiment using fresh membranes. This profile of staining indicates that the AP activity is membrane-associated, and can be released by PI-PLC. The decreased mobility of the membrane-bound AP activity in lane A, as compared with the bands in lanes D and E, may be due to multimerization or association with lipids and/or other membrane proteins. The bands of AP activity in lanes D and E are diffuse, precluding the identification of a single AP species in the membrane preparations using this technique.

grasshopper membrane band were recognized by normal mouse serum.

e-AP expression and migration of neuronal growth cones

Pathfinding by growth cones of the Ti1 afferent pioneer neurons in grasshopper limb buds has been extensively studied (Bate, 1976; Bentley and O'Connor, 1991; Jay and Keshishian, 1990; Kolodkin et al., 1992). It has been demonstrated that the epithelial substrate provides sufficient information for pioneer growth cone guidance (Condic and Bentley, 1989b; Lefcort and Bentley, 1987). Consequently, the relationship between pioneer axon trajectories in the limb and bands of e-AP-expressing epithelial cells was examined by double-labeling limbs with neuron-specific (anti-HRP) antibodies.

The Ti1 pioneer neurons are sibling cells that arise from an epithelial precursor cell at the tip of the limb bud at about the 30% stage of embryogenesis and project axons proximally along the limb axis toward the central nervous system. The Ti1 cell bodies are located within the broad band of e-AP-expressing epithelial cells at the tibia-femur segment boundary (Fig. 7); their growth cones emerge onto an epithelial surface that strongly expresses e-AP. As they migrate proximally, the Ti1 growth cones show no consistent change in morphology at the proximal border of the e-AP band in the mid-femur.

Within the trochanter, the Ti1 growth cones normally undergo a marked reorientation (Caudy and Bentley, 1987; Kolodkin et al., 1992; O'Connor et al., 1990). They cease proximal migration, extend processes dorsally and ventrally

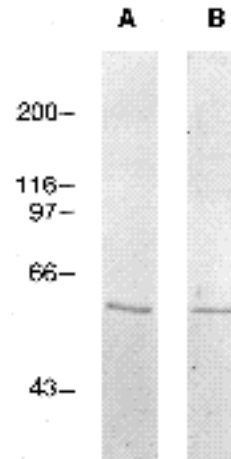


Fig. 6. Probe of embryonic grasshopper membrane protein with anti-AP serum antibody. An immunoblot of 0.15 µg of shrimp AP (lane A) and 100 µg of embryonic grasshopper membrane protein (lane B) was probed with a polyclonal anti-shrimp AP antiserum. The antiserum recognized the $55 \times 10^3 M_r$ shrimp AP as well as a single prominent $56 \times 10^3 M_r$ band of grasshopper membrane protein. The molecular mass standards ($\times 10^{-3}$) migrated as indicated.

along the limb circumference, and subsequently make a sharp ventral turn. Double-labeling of limbs with AP substrates and anti-HRP antibodies shows that this reorientation occurs at the proximal edge of the e-AP band that spans the femur-trochanter segment boundary (Fig. 7A), and that the growth cones subsequently migrate ventrally along the edge of this band (Fig. 7B). Examination at different focal planes (Fig. 7C,D) of double-labeled metathoracic limbs suggests that the growth cones normally migrate along the interface between the e-AP expressing cells and the adjacent domain of non-expressing cells, and primarily overlie the most distal tier of non-e-AP expressing cells. In all double-labeled limbs examined between the 33-34.5% stages of embryogenesis, the location of the ventral turn by the Ti1 growth cones was always within one cell diameter's width of the proximal edge of e-AP expression in the trochanter ($n=38/38$).

DISCUSSION

Alkaline phosphatases (APs; E.C. 3.1.3.1; phosphomonoester hydrolases) are a small family of highly conserved and phylogenetically widely distributed cell surface and secreted metalloenzymes. Many are GPI-anchored glycoproteins, and all occur as multimers and require divalent cations for activity (McComb et al., 1979). The structure of *E. coli* AP has been described at 2.8 Å resolution, and the active site and two zinc binding sites are well characterized (Sowadski et al., 1985). Between bacterial (*E. coli*), insect (*Bombyx mori*), and human AP forms, amino acid identity is fully conserved through all three sites (Itoh et al., 1991).

Four isozymes occur in humans (Hahnel et al., 1990; Harris, 1989). Intestinal (I-AP), placental (PI-AP) and germ cell (GC-AP) APs constitute a tissue-specific subfamily, and are approximately 90% homologous to each other at

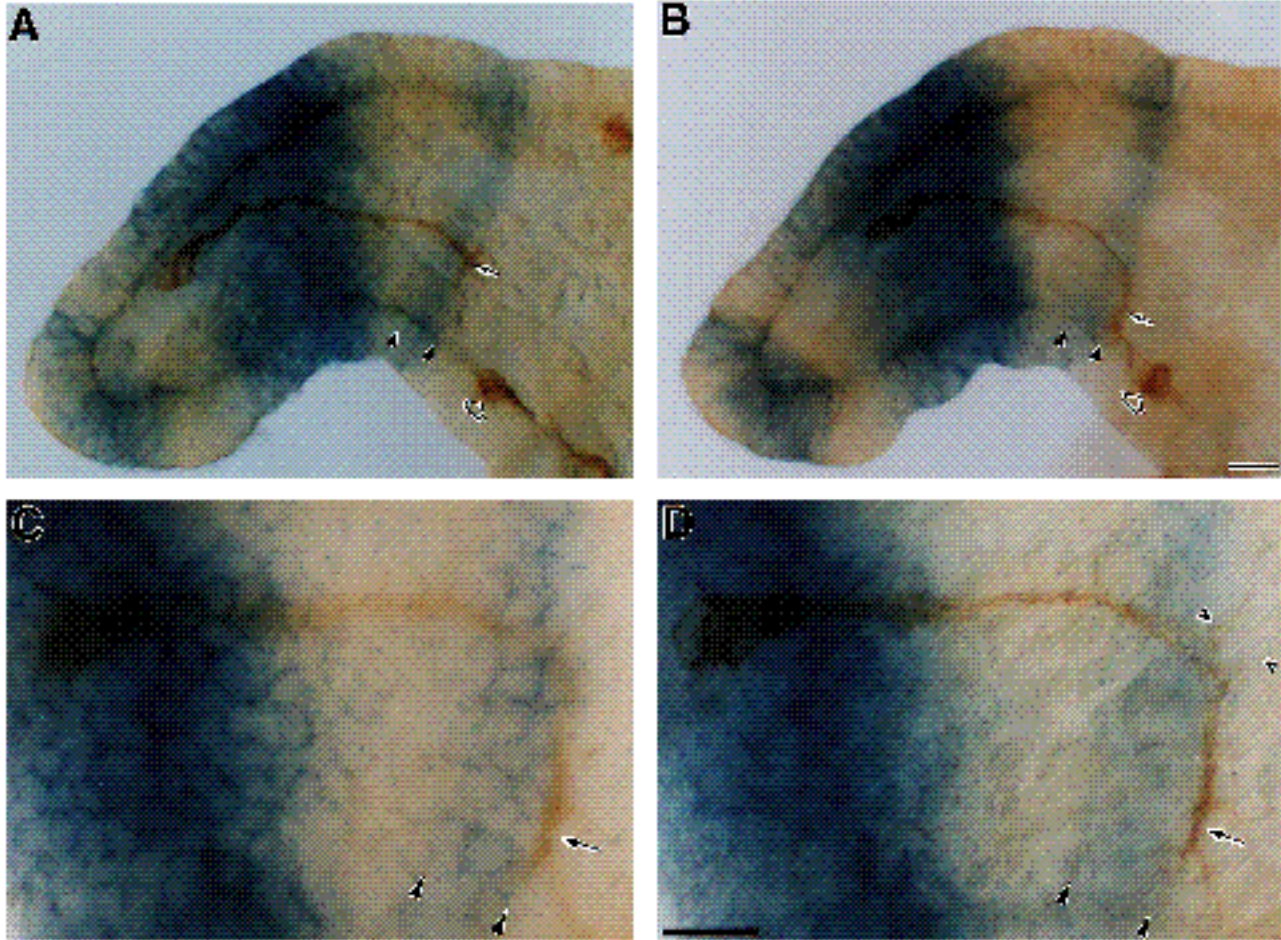


Fig. 7. The stereotypic ventral turn made by Ti1 pioneer growth cones within the trochanter is tightly correlated with the boundary of one AP-expressing epithelial domain (Ti1 pathway reviewed in Bentley and O'Connor, 1991). (A-D) Photomicrographs of three different limbs double-labeled for AP activity (blue) and with neuron-selective (anti-HRP) antibodies (brown). (A) Upon reaching the proximal edge of the femur-trochanter band of e-AP activity, the Ti1 growth cones (arrow) extend circumferential processes dorsally and ventrally along the epithelium in the trochanter (33% stage prothoracic limb; open arrow, Cx1 guidepost cells). (B) After turning ventrally within the trochanter, the Ti1 growth cones (arrow) migrate along the edge of the e-AP band at the femur-trochanter segment boundary before turning proximally toward the Cx1 guidepost cells (open arrow; 34% stage mesothoracic limb). (C, D) Photomicrographs of a 33.5% stage metathoracic limb at focal planes within the anterior epithelium (C), and at the basal surface of the epithelium (D). In C, the plane of focus reveals the AP-expressing epithelial band at the femur-trochanter boundary, about three cells in width; the relative location of the pioneer growth cones (arrow) is visible just out of the focal plane. In D, the focal plane shows the Ti1 cell bodies, axons, and growth cones. The growth cones (arrow) migrate along the proximal edge of the AP epithelial domain; they appear to overlie the most distal tier of non-AP expressing epithelial cells (caretts: Tr1 guidepost cell). (A-D) Distal, to left; dorsal, up. In each panel, the pair of arrowheads indicates the distal and proximal edges of the femur-trochanter e-AP band. Scale bars, 25 μm (A, B on B; C, D on D).

the amino acid level. A tissue non-specific form (TN-AP) is found in liver, bone, and kidney, and is approx. 57% homologous to the tissue-specific APs. In *Drosophila* and other insects, at least two isozymes occur (Psarianos et al., 1987). The membrane-associated form of silkworm (*Bombyx mori*) AP (m-AP) is the only insect AP for which the nucleotide sequence has been determined. It is a 511 amino acid protein with a calculated M_r of 56×10^3 , and is GPI-anchored to midgut epithelial cells (Itoh et al., 1991). It has 44.6% overall amino acid identity with human TN-AP, and complete identity in the active site and zinc binding sites.

Preliminary biochemical analyses suggest that the grasshopper e-AP is similar to silkworm AP, as well as to other insect and arthropod APs. The grasshopper enzyme

is defined as an alkaline phosphatase by its ability to hydrolyze chromogenic phosphatase substrates at alkaline pH (Wolf et al., 1967). Like the epithelial AP of the Mediterranean fruit fly (Psarianos et al., 1987) and of *D. melanogaster* (Harper and Armstrong, 1972), it is active over a fairly broad range of pH conditions (Fig. 4C). It is GPI-anchored (Figs 4B, 5), as are APs from silkworm (m-AP; Takesue et al., 1989), from the moth (*Trichoplusia*) TN-368 cell line (Ikezawa et al., 1989), and from shrimp (*Penaeus japonicus* Bate) hepatopancreas (Chuang, 1990). An antiserum raised against the $55 \times 10^3 M_r$ shrimp AP recognizes a single band at $56 \times 10^3 M_r$ on immunoblots of embryonic grasshopper membrane proteins (Fig. 6). While the similarity between this value and the M_r reported for AP enzymes from shrimp (Chuang, 1990) and silkworm

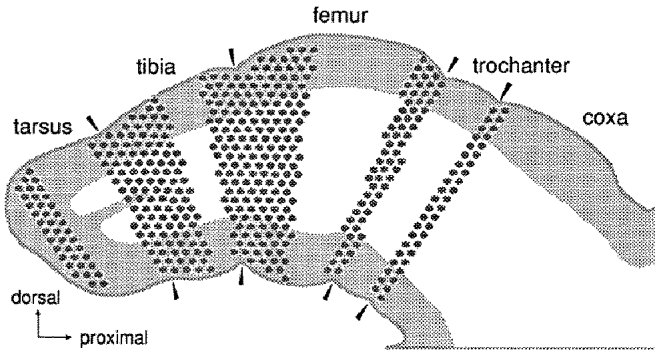


Fig. 8. A schematic diagram depicting the five circumferential bands of e-AP-expressing epithelial cells in a 36% metathoracic grasshopper limb. At this stage, a band spans each intersegmental boundary within the limb; the remaining band lies within the tarsus. Hexagons indicate the locations of the bands, and their width in epithelial nuclear diameters (because the epithelial nuclei are packed in two tiers, the bands comprise more epithelial cells than is indicated by their width). Arrowheads indicate developing segment boundaries.

(Itoh et al., 1991) is suggestive, this $56 \times 10^3 M_r$ band has not been shown directly to have AP activity. On non-denaturing gels, the grasshopper e-AP runs as a single broad band (Fig. 5). A single broad band is also seen on non-denaturing gels of shrimp hepatopancreas (Chuang, 1990), and adult cockroach brain (Verhaert et al., 1990) stained for enzymatic activity; higher resolution gels suggest that at least two AP bands of similar mobility are present in the cockroach (Verhaert et al., 1990). It appears likely from these results that the grasshopper e-AP is a single protein, or a small group of isozymes, which are similar in electrophoretic mobility.

Although grasshopper e-AP is likely to share the highly conserved active site and zinc binding domains of AP (Itoh et al., 1991), APs do vary in susceptibility to inhibitors. A series of compounds that have been shown to inhibit the activity of various AP isozymes were assayed for effect on e-AP (Table 1). Divalent cations, particularly Mg^{2+} , are required for complete activation of many APs (McComb et al., 1979); as expected, 10 mM EDTA significantly reduced e-AP activity. However, levamisole and tetramisole, potent inhibitors of several tissue non-specific APs, had little effect on the grasshopper e-AP in appropriate concentration ranges. Similarly, exposure to L-phenylalanine, which inhibits several intestinal and placental AP isoforms, did not diminish e-AP activity. Consequently, there are differences between grasshopper e-AP and some other characterized APs.

The most potent inhibitors of grasshopper e-AP activity were nucleoside monophosphate compounds, such as AMP and IMP (Fig. 4D) and sodium orthovanadate. Since the non-phosphorylated nucleosides adenosine and inosine were ineffective inhibitors, the presence of the phosphate group is required. The nucleotide compounds may serve as substrates for phosphatase activity and could exert their inhibitory effect through competition for active sites on the enzyme molecule. This leads to the possibility that the embryonic grasshopper AP could function as a nucleotidase

in vivo, dephosphorylating nucleoside phosphates in the extracellular space. At least one other AP enzyme, a soluble AP from the midgut of the silkworm, *Bombyx mori*, functions as an ATPase in vitro (Azuma et al., 1991). Ectonucleotidase activities have been demonstrated in invertebrate (Gleeson et al., 1989) and vertebrate (Low and Finean, 1978) tissues and, like APs, can be GPI-anchored (Low and Finean, 1978).

e-AP expression and limb morphogenesis

Limb transplantations (Bohn, 1970, 1971; French, 1981), and experiments with serum antibodies generated against portions of limb segments (Bulliere et al., 1982) have suggested that an important element of limb segment morphogenesis may be the repetition within each primary segment of positional fields of information. If these fields reflect molecular differentiation, such molecules should be expressed in repeated patterns along the limb. Two such patterns have recently been identified: the DSS-8 antigen, a $164 \times 10^3 M_r$ glycoprotein (Wang et al., 1992), is expressed in the distal portion of each limb segment during cockroach limb morphogenesis (Norbeck and Denburg, 1990). In the grasshopper embryo, an apparently corresponding set of cells expresses annulin, a $97 \times 10^3 M_r$ protein homologous to transglutaminases (Bastiani et al., 1992; Singer et al., 1992). Fasciclin IV, a novel transmembrane glycoprotein of $80 \times 10^3 M_r$, is expressed in five, segmentally iterated, intrasegmental, circumferential epithelial bands during grasshopper limb development (Kolodkin et al., 1992). The pattern of e-AP expression molecularly defines a third segmental epithelial domain (Fig. 8), supporting the view that segmentally iterated molecular information is an important element of limb segmentation. The bands of e-AP expression are distinct from those of DSS-8, annulin and fasciclin IV, in that they span boundaries between limb segments. With the identification of several molecular markers for grasshopper limb epithelium, it will now be possible to investigate the temporal and spatial relationships between these epithelial domains within single limb segments.

Possible functions of grasshopper e-AP

Physiological functions of APs currently are not well understood (Harris, 1989; McComb, 1979). During embryogenesis in a variety of species, expression often appears to be spatially and temporally restricted to populations of cells that are migrating, or undergoing morphogenesis (Karczmar and Berg, 1951; McAlpine, 1956; McWhinnie and Saunders, 1966; Zackson and Steinberg, 1988). In the early mouse embryo, AP is expressed on migrating primordial germ cells (Chiquoine, 1954; Mintz and Russell, 1957) and may be involved in dissociation of trophectoderm from the inner cell mass (Hahnel et al., 1990). In embryos of the axolotl, *Amblystoma mexicanum*, distribution of AP activity and effects of treatment with both levamisole and PI-PLC suggest that AP may guide migration of pronephric duct cells, possibly through participation in an adhesive mechanism (Zackson and Steinberg, 1988, 1989).

In appendages of the embryonic grasshopper, the spatially restricted expression of e-AP suggests that it may

function in epithelial morphogenesis. It is expressed near the tips of limb buds (Fig. 2A), at limb and antennal segment boundaries (Figs 1, 2E), and along epithelial apodemes (Fig. 2E). All of these are regions of epithelial shape changes, including limb bud evagination, segment boundary invagination, and apodeme extension into the limb interior. The tibia and femur, where e-AP bands are broadest (at the onset of limb segmentation), are the segments that undergo the most extensive elongation during limb morphogenesis. In insects, epithelial shape changes may involve both changes in cell shape and cell rearrangements (Condic et al., 1991). Extracellular enzymes, like e-AP, could mediate cell adhesion upon the disassembly of more permanent epithelial junctions, or, alternatively, could contribute to a reduction of epithelial adhesion which permits rearrangement. The pattern of expression of e-AP in grasshopper limb buds is consistent with a potential function in the interactions of epithelial cells with neighboring cells or the extracellular matrix that occur during cell movements and rearrangements in limb morphogenesis. Culturing embryos in the presence of sodium orthovanadate at concentrations sufficient to nearly completely inhibit histochemical staining in fixed tissue led to reproducible defects in limb morphogenesis, including the incomplete elaboration of segment boundaries (data not shown). However, because vanadate is a broad specificity phosphatase inhibitor that may enter cells and affect intracellular signaling events, the observed effects cannot be solely attributed to its inhibition of e-AP activity. More specific reagents will be required to unequivocally assign a functional role for the e-AP activity in grasshopper limb development.

e-AP expression and neuronal pathfinding

Pathfinding by Ti1 pioneer growth cones in insect limb buds has been extensively studied (Bate, 1976; Bentley and O'Connor, 1991; Jay and Keshishian, 1990; Wang and Denburg, 1991). In grasshoppers, a prominent event in the migration of these growth cones is the abrupt turn from proximal growth to ventral, circumferential growth along a band of fasciclin IV expressing epithelial cells within the trochanter (Caudy and Bentley, 1987; Kolodkin et al., 1992; O'Connor et al., 1990). This steering event can be mediated by growth cone interactions with cells of the epithelial substrate (Condic and Bentley, 1989b; Lefcort and Bentley, 1987). When limbs are treated with PI-PLC before the arrival of growth cones in the trochanter, a significant proportion of growth cones fail to make this turn, and cross the trochanter-coxa boundary into the coxa (Chang et al., 1992).

This result suggests that GPI-anchored molecules on the growth cones and/or the substrate of epithelial cells are important in mediating this steering event. Two GPI-anchored molecules, fasciclin I (Bastiani et al., 1987) and lachesin (Karlstrom and Bastiani, 1992; Karlstrom et al., 1993) are expressed on the Ti1 growth cones. Functional inactivation experiments on lachesin have not yet been reported. Chromophore-assisted laser inactivation of fasciclin I results in defasciculation of the Ti1 axons, but does not effect the ventral turn in the trochanter (Jay and Keshishian, 1990). Thus, fasciclin I does not appear to be

a GPI-anchored molecule whose inactivation results in trochanter-coxa boundary crossing.

If a GPI-anchored molecule on the epithelial substrate is involved in guiding Ti1 growth cones in the trochanter, then a discontinuity in its distribution might be observable at the axial location where the growth cones normally reorient from axial to circumferential growth. Fasciclin IV is selectively expressed on the band of epithelial cells on which the Ti1 growth cones turn (Kolodkin et al., 1992), and is important for normal growth in the trochanter. However, it is not GPI-anchored, and blocking with antibodies (Kolodkin et al., 1992) does not mimic the boundary crossing phenotype (Chang et al., 1992) produced by PI-PLC treatment.

The results presented here show that e-AP is a substrate-bound, GPI-anchored cell surface molecule whose expression marks the location of the Ti1 growth cone turn within the trochanter. In embryos labeled with neuron-specific antibodies and stained for e-AP, the Ti1 growth cones are seen to turn at the interface between the band of e-AP cells and more proximal, non-AP expressing cells within the trochanter (Fig. 7). Consequently, e-AP is a candidate for a GPI-anchored molecule whose removal results in failure of Ti1 growth cones to respond normally to guidance signals within the trochanter. e-AP might function in guidance either enzymatically, by dephosphorylating a substrate on the growth cone, or non-enzymatically, as an epithelial surface recognition ligand. Although the developmental roles of alkaline phosphatases are not well understood, they, and other cell surface ectoenzymes (Begovac and Shur, 1990), may be important in cell adhesion, rearrangement, migration and guidance (Zackson and Steinberg, 1989).

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