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Original Article

The expression of MDP-1, a component of *Drosophila* embryonic basement membranes, is modulated by apoptotic cell death

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ABSTRACT Using a novel monoclonal antibody we have studied the expression of a large proteoglycan-type molecule in *Drosophila* embryos. This molecule is secreted exclusively by migratory, embryonic hemocytes/macrophages and was therefore named MDP-1 for Macrophage-Derived Proteoglycan-1. Expression of MDP-1 begins late during hemocyte differentiation, after these cells have left their birthplace in the head mesoderm. At this time, macrophages are engaged in extracellular matrix deposition and the phagocytosis of cell debris generated by apoptotic events in various parts of the embryo, in particular from the developing central nervous system. Embryos deficient for programed cell death display a greatly reduced amount of MDP-1 deposition in tissues that normally undergo morphogenetic cell death. This suggests a regulatory role for apoptosis in the terminal differentiation of *Drosophila* hemocytes. MDP-1 is initially deposited around the developing central nervous system and is later found in basement membrane structures surrounding various other organs, such as the gut, Malpighian tubules and part of the tracheal system. The temporal and localized deposition of MDP-1 suggests that it may play a role in delineating the central nervous system structure during axonogenesis and may participate in the formation of a functional 'blood-brain barrier' in *Drosophila*.

KEY WORDS: *apoptosis, Drosophila, hemocytes, proteoglycan, development*

Introduction

Proteoglycans constitute a diverse group of macromolecules which are composed of a protein core with a varying number of covalently attached, sulfated glycosaminoglycan (GAG) side chains (Ruoslahti, 1988; Kjellen and Lindahl, 1991). Various types of proteoglycans are present on cell surfaces and in the extracellular matrix. Proteoglycans are not only structurally important molecules, they also have many additional functions and are essential for the proper differentiation of various tissues and organs. The expression and the functional role of proteoglycans in the developing nervous system have been studied in some detail (Lander, 1993; Letourneau *et al.*, 1994; Margolis and Margolis, 1994). Numerous reports have demonstrated that several different proteoglycans inhibit neural adhesion and neurite outgrowth (Snow *et al.*, 1990; Oohira *et al.*, 1991), whereas other proteoglycans appear to promote neurite outgrowth (Lander *et al.*, 1982; Matthew *et al.*, 1985; Faissner *et al.*, 1994). These sometimes opposing effects of different proteoglycans on differentiating neurons may be mediated in part by their direct interactions with several neural cell adhesion molecules such as NCAM, L1-CAM, and cytotactin (Cole *et al.*, 1986; Hoffman and Edelman, 1987; Friedlander *et al.*, 1994; Maurel *et al.*, 1994) as well as other extracellular matrix molecules such as laminin, collagen and fibronectin (Oldberg and Ruoslahti, 1982; Lander *et al.*, 1985; Saunders and Bernfield, 1988). It has been hypothesized that proteoglycans may provide either permissive or inhibitory cues for many cell-cell and cell-substrate interactions during nervous system development. Acting through these mechanisms, proteoglycans could have an instructive influence on developing patterning processes like axonal pathway choices.

Most of the above studies were performed using *in vitro* assay systems and no systematic attempts have been made to study the developmental role of proteoglycans in a genetic system. For

Abbreviations used in this paper: CNS, central nervous system; HRP, horseradish peroxidase; MDP-1, macrophage-derived proteoglycan-1; PBS, phosphate-buffered saline.

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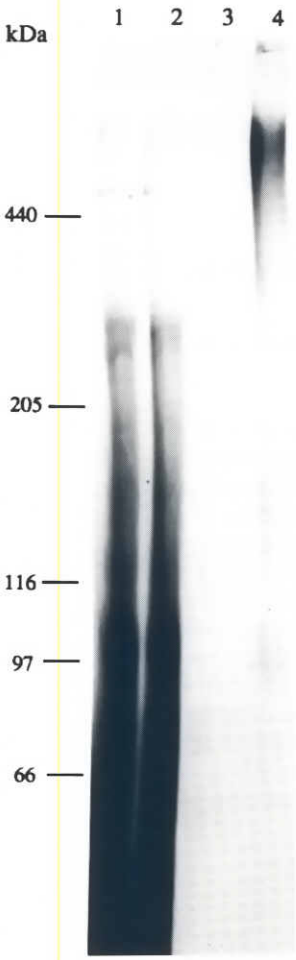

Fig. 1. Immunoaffinity purification of embryonic MDP-1. *An affinity column was prepared by crosslinking the 5H7 monoclonal antibody to a Protein G-Sepharose 4 Fast Flow matrix. Total protein extracts from Drosophila embryo homogenates were passed over the column and aliquots of purified 5H7 antigen were eluted using a high pH buffer. Fractions containing 5H7 antigen were separated on 3 to 10% gradient SDS-polyacrylamide gel and stained with silver. From left to right:* **Lane 1** *represents the total embryonic protein extract used as starting material,* **lane 2** *shows the flow through protein pattern,* **lane 3** *the preelution fraction, and* **lane 4** *the MDP-1 containing eluate.*

example, very few proteoglycans have been identified and further studied in the fruitfly *Drosophila melanogaster*. Various types of sulfated and unsulfated proteoglycans are present in many different species throughout various invertebrate phyla (Höglund, 1976; Cassaro and Dietrich, 1977), where they appear to fullfill similarly important roles as their counterparts in vertebrates. Most studies in *Drosophila* have been either based on monoclonal antibodies which are specific for fly proteoglycans (Brower *et al.*, 1987; Graner *et al.*, 1994), the biochemical isolation of a proteoglycan-like molecule from a *Drosophila* cell (Campbell *et al.*, 1987), or the cDNA cloning of a fruitfly homolog of the well characterized vertebrate membrane-associated proteoglycan syndecan (Spring *et al.*, 1994). The only genetic study of a *Drosophila* proteoglycan has been presented by Nakato *et al.* (1995). They identified the gene product of the *division abnormally delayed (dally)* locus as a glypican-related proteoglycan. This GPI-anchored membrane proteoglycan appears to be required for the proper progression of cell division in the eye imaginal disc and the optic lamina during postembryonic development. However, the developmental expression patterns of specific proteoglycans and their potential roles in embryonic development have not been analyzed to any detail in *Drosophila*.

The major source for extracellular matrix molecules in the *Drosophila* embryo are migratory hemocytes (Fessler *et al.*, 1994). These cells are derived from the procephalic mesoderm and start migrating throughout the embryo along characteristic routes during stage 11 of embryonic development (Tepass *et al.*, 1994). Hemocytes produce a wide range of different extracellular matrix molecules including Laminin, Type IV Collagen, Peroxidasin, Tiggrin and Glutactin (Fessler *et al.*, 1994). By secreting these extracellular matrix molecules, they contribute to the formation of basement membranes in the developing embryo. In addition to their biosynthetic function, *Drosophila* hemocytes are actively involved in wound healing, capsule formation and the phagocytosis of foreign particles and cell debris (Brehelin, 1982). Phagocytic hemocytes can therefore be considered functional homologs of vertebrate macrophage cells.

Here we characterized a large, highly glycosylated *Drosophila* antigen, called MDP-1 for Macrophage-Derived Proteoglycan. MDP-1 may be related to a proteoglycan-like molecule, called Papilin, which had been previously isolated from a *Drosophila* cell line (Campbell *et al.*, 1987). Using *Drosophila* mutant and enhancer trap lines we demonstrate that MDP-1 is secreted by differentiated hemocytes and is preferentially deposited in embryonic basement membranes, especially the neural lamella surrounding the developing central nervous system. MDP-1 is first expressed by hemocytes after they have migrated throughout the embryo and have encountered cell debris generated by apoptotic cell death. Apoptosis is not absolutely required for MDP-1 expression to occur. However, tissues with high levels of developmental programed cell death, such as the nervous system, the head region and midgut, have high levels of MDP-1 deposition. In contrast, reduced levels of MDP-1 expression were observed in embryos deficient for apoptosis.

Results

Biochemical and immunological characterization of the MDP-1 in Drosophila embryos

Antibodies raised against membrane proteins present on *Drosophila* primary adherent cells have provided useful tools for the

TABLE 1

CARBOHYDRATE COMPOSITION OF PURIFIED MDP-1 PREPARATIONS

Carbohydrate	embryonic MDP-1 µg/mg protein	Kc cell Papilin µg/mg total amino acids
Neutral sugars	101 ± 24	270
Uronic acids	136 ± 19	610
Amino sugars	577 ± 42	950
Total carbohydrate ± SEM	815 ± 77	1,830

Protein was determined by the method of Lowry (1951), neutral sugars by the phenol-sulfuric acid method of Dubois *et al.* (1956), uronic acids by the carbozole reaction of Bitter and Muir (1962) and amino sugars by the modified Elson-Morgan reaction of Augustyniak and Augustyniak (1966). The second column displays the carbohydrate content of Papilin as reported by Campbell *et al.* (1987).

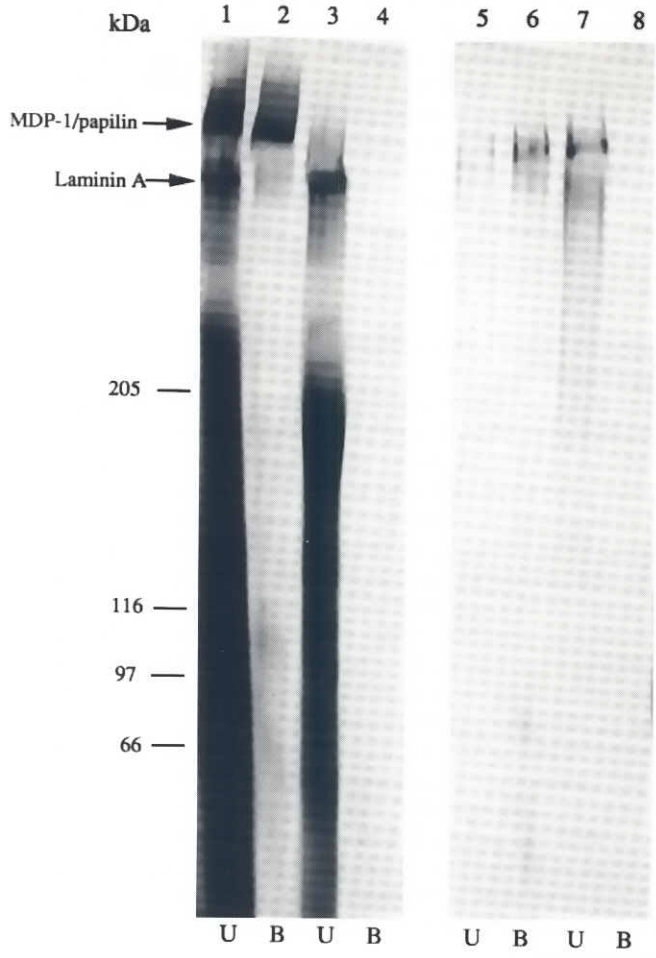

Fig. 2. The 5H7 mAb immunoprecipitates *Drosophila* Kc cell Papilin. *5H7 mAb was bound to Protein G-Sepharose 4 Fast Flow beads and covalently crosslinked to the matrix. After a preelution step 5H7 or control beads were incubated with protein extracts as indicated and subsequently washed before analyzed on 3 to 5% gradient SDS-polyacrylamide gels. Bound proteins were visualized by silver staining. Odd numbered lanes which are denoted with the letter 'U' display proteins which did not bind to the 5H7 antibody affinity matrix. Even numbered lanes which are denoted with the letter 'B' display the corresponding proteins which did bind to the 5H7 mAb matrix.* **Lanes 1 and 2** *depict a 5H7 immunoprecipitation from a Papilin-enriched fraction isolated from K2 cells. This protein mixture contains Papilin, Laminin and other extracellular matrix molecules. A different, lighter protein fraction from the same gradient does not contain Papilin is shown in* **lanes 3 and 4. Lanes 5 and 6** *represent an immunoprecipitation of affinity purified 5H7 antigen from Drosophila embryos using the 5H7 antibody matrix.* **Lanes 7 and 8** *show a control precipitation using a monoclonal antibody of the same subclass as the 5H7 antibody which fails to precipitate affinity purified 5H7 antigen.*

identification and initial characterization of proteins involved in cellular interactions (Hortsch *et al.*, 1990a,b). Relatively few *Drosophila* embryonic cell types, mainly of mesodermal and neuroectodermal origin, adhere to tissue culture plastic in short term cultures (Cross and Sang, 1978). We used membrane preparations isolated from such cells to immunize mice for the production of monoclonal antibodies (mAbs). Spleen cells of immunized mice were fused to myeloma cells and hybrid cells were tested for the production of antibodies giving interesting staining patterns in *Drosophila* embryos using whole-mount immunocytochemistry. One hybrid cell line, called 5H7, was retained and used for the biochemical and developmental characterization of the antigen recognized by this mAb. The 5H7 antibody stained a subset of cultured, adherent embryonic cells which had a round cell morphology and were usually associated with smaller groups of unstained adherent cells (data not shown). Since the 5H7 mAb did not react with any *Drosophila* protein on Western blots, we used an immunoaffinity purification procedure to identify the antigen recognized by this mAb. A single large protein band with an apparent molecular weight of approximately 800 kDa was purified from total *Drosophila* embryonic protein extracts (Fig. 1). Its electrophoretic behavior suggested that this protein band might be heavily glycosylated and a carbohydrate analysis showed that the protein component accounts for less than 50% of the molecule (Table 1). With amino sugars being the most abundant type of carbohydrate subunit, the presence of uronic acid indicated that the 5H7 antigen is a proteoglycan-type macromolecule. An amino acid analysis of purified 5H7 antigen revealed an abundance of acidic and hydroxyl amino acid residues (Table 2). Although some significant differences are apparent, the size and overall amino acid profile resembles that of a proteoglycan-like molecule which had been previously isolated from *Drosophila* Kc cell-conditioned medium and referred to as Papilin (Campbell *et al.*, 1987; Table 2). Figure 2 shows that our 5H7 mAb specifically immunoprecipitated this Kc cell-derived Papilin out of a protein mixture of different *Drosophila* ECM molecules. Since the 5H7 mAb does not immunoprecipitate denatured MDP-1 or Papilin and does not recognize any *Drosophila* polypeptide on Western blots, its epitope is most likely formed by the protein rather than the carbohydrate portion of the molecule. Although our biochemical analysis of the 5H7 antigen, which we purified from *Drosophila* embryos, revealed several significant differences to the Papilin molecule, it is possible that it is the naturally occurring form of Papilin, which is secreted by *Drosophila* Kc cells in culture.

Migratory hemocytes express MDP-1 after they have left the procephalic mesoderm

The staining pattern of 5H7 positive cells agrees well with the presence of migratory hemocytes. These cells are derived from the procephalic mesoderm (Tepass *et al.*, 1994). An early marker for this cell lineage in *Drosophila* is the extracellular matrix molecule Peroxidasin (Nelson *et al.*, 1994). Peroxidasin is first expressed at about stage 10 in the embryonic head region (Fig. 3A). As described by Tepass *et al.* (1994), during embryonic stage 11 hemocytes start migrating posteriorly along the dorsal surface into the tip of the extended germ band and ventrally along the forming ventral nerve cord. At this stage 5H7 immunostaining is first detected along the dorsal side of the developing anterior ventral nerve cord and the posterior spiracle primordium in the tip of the extended germ band (Fig. 3C). In contrast to Peroxidasin, no 5H7 immunostaining is detected in the head region, where hemocytes initially differentiate, prior to developmental stage 11 (Fig. 3B). During embryonic stage 12 5H7-positive cells have migrated along the entire length of the ventral nerve cord and have entered the areas between the anterior and posterior commissures of adjacent segments (also called midline pores) (Fig. 4A). 5H7 antigen

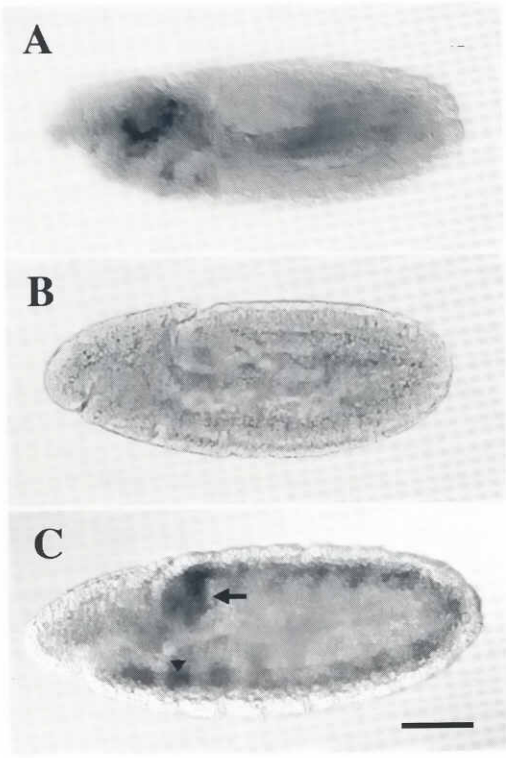

Fig. 3. Whole-mount staining for MDP-1 and Peroxidasin of early *Drosophila* embryos. *Drosophila embryos were fixed and processed for immunocytochemistry as described in the Materials and Methods section.* **(A)** *shows an early stage 11 embryo which has been stained with a polyclonal anti-Peroxidasin antiserum.* **(B and C)** *depict embryos of early and late developmental stage 11, respectively, which were stained using the 5H7 mAb. The arrow in panel C indicates 5H7 staining at the tip of the extended germ band. Immunostaining along the anterior dorsal surface of the differentiating ventral nerve cord is marked by an arrowhead. Bar, 100 μm.*

becomes deposited in a membranous structure surrounding the developing neuropile (indicated by arrowheads in Fig. 4A). This membrane structure appears to be identical with the previously described neural lamella, which constitutes part of the insect 'blood-brain barrier' (Juang and Carlson, 1994). 5H7-positive cells are now also found in the space between the ventral nerve cord and the underlying ectoderm. At the end of embryonic stage 11, 5H7 staining also becomes apparent in the head region around the developing brain lobes and in the clypeolabrum.

At this developmental stage the cellular staining patterns for Peroxidasin and for the 5H7 antigen are very similar (Fig. 4B and C). This suggests that the antigen recognized by the 5H7 mAb is either made by or is taken up by migratory hemocytes and we will therefore subsequently use the term MDP-1 (Macrophage-Derived Proteoglycan-1) for the macromolecule recognized by the 5H7 mAb.

MDP-1 is specifically expressed by hemocytes in the Drosophila embryo

In order to demonstrate that 5H7 staining coincides with hemocytes and that MDP-1 is produced by these cells rather than taken up by phagocytosis, we examined a hemocyte-specific enhancer trap line and mutant *Drosophila* embryos which do not develop cells of the mesodermal hemocyte lineage.

The *A109.1F2* enhancer trap line contains a P-element with a β-galactosidase gene near the enhancer of the *Drosophila* collagen IV gene on the second chromosome (Wilson *et al.*, 1989). β-galactosidase protein in this line is expressed in differentiated hemocytes and can be detected cytochemically as a blue nuclear stain using X-gal as a substrate. *A109.1F2* embryos were first stained with X-gal (visible as a dark blue, nuclear staining) and subsequently incubated with 5H7 mAb, followed by HRP-conjugated anti-mouse IgG secondary antibody and developed with DAB as the substrate (5H7 immunostaining is visible in brown). In these embryos cellular 5H7 stain coincided with nuclear β-galactosidase staining. This is especially conspicuous for hemocyte cells along the ventral nerve cord as demonstrated in Figure 5. This result confirms that MDP-1 is highly enriched in *Drosophila* embryonic hemocytes.

That MDP-1 is produced by these cells, rather than taken up by phagocytosis, was demonstrated using different mutant *Drosophila* lines which are lacking specific embryonic structures or tissues. Embryonic hemocytes are derived from a small number of mesodermal cells in the cephalic region of the embryo (Tepass *et al.*, 1994). Embryos which are derived from *Bic-D* mutant mothers are missing anterior structures and as a result are unable to produce and differentiate cells of the hemocyte lineage. No staining was visible when embryos exhibiting a strong *Bic-D* phenotype were stained using the 5H7 mAb, indicating that the 5H7-positive cells lining the nervous system and other developing organs are derived

TABLE 2

Amino acid analysis of purified MDP-1 protein preparations isolated from *Drosophila* embryos

	Ser	Glu	Gly	Asp	Thr	Ala	Lys	Pro	Leu	Val	Arg	Ile	Tyr	Phe	Met
embryonic MDP-1	159	138	127	104	87	73	54	52	48	42	32	23	21	17	6
Kc cell Papilin	125	121	92	124	92	65	27	72	37	45	38	35	19	23	6

# of residues per 1000 amino acid residues

Amino acid analysis was performed by the University of Michigan Biomedical Research Core Facilities and carried out after automated acid hydrolysis on an ABI model 429H amino acid analyzer by the PITC method. The His and Cys content of MDP-1 was not analyzed. Data for Kc cell Papilin are shown as reported by Campbell et *al.* (1987).

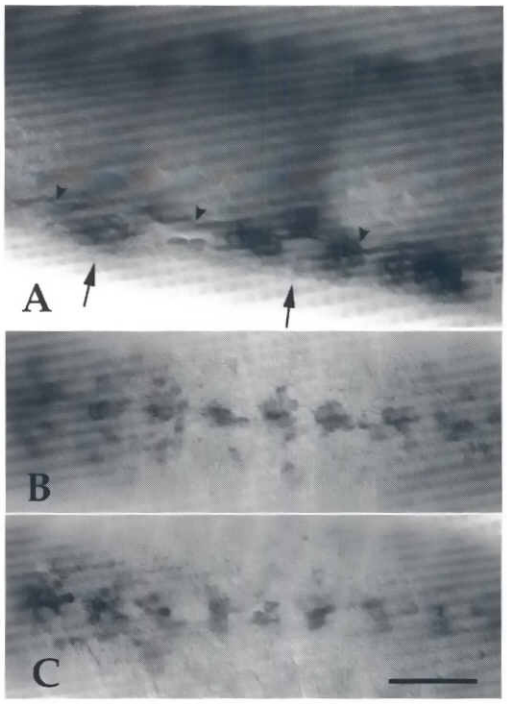

Fig. 4. Anti-MDP-1 staining is prominent in migratory cells along the developing ventral nerve cord in *Drosophila* embryos. *Drosophila embryos were collected and processed for immunocytochemistry using either the anti-MDP-1 mAb 5H7* **(A and B)** *or a mouse anti-Peroxidasin antiserum* **(C).** *Panel A shows a sagittal view of the developing nerve cord of a stage 13 Drosophila embryo. The anterior end of the embryo is to the left. The arrows indicate the segmental pattern of the midline pores between the posterior commissure and the anterior commissure of the next posterior segment. Arrowheads indicate an acellular, MDP-1-positive membrane which surrounds the developing neuropile and is presumably identical with the previously described neural lamella (Edwards et al., 1993; Juang and Carlson, 1994). Panels B and C show ventral views of stage 13 embryos with the anterior ends to the right. Bar, (A) 20 μm, (B) and (C) 60 μm.*

from anterior tissues (Fig. 6A). Similar results were obtained for *Bic-D* embryos stained with an anti-Peroxidasin antiserum (Nelson *et al.*, 1994; data not shown).

Snail-twist-deficient embryos fail to develop all mesodermal tissues, including the hemocyte anlage. No 5H7 staining was observed in sna^{-}-twi^{-} mutant embryos (Fig. 6B). Although the *Bic-D* as well as the *snail-twist* mutations affect a number of different tissues and developmental processes, these results support that differentiated embryonic hemocytes are the sole source of MDP-1 during early *Drosophila* embryogenesis.

MDP-1 is deposited in basement membranes during late Drosophila embryogenesis

During later stages, migrating hemocytes are found throughout the *Drosophila* embryo and play an important function in basement membrane biogenesis. From embryonic stage 13 on, MDP-1 staining becomes increasingly stronger throughout the embryo, especially in areas where basement membranes are forming. 5H7 staining is most concentrated around several developing organs, such as the surface of the brain hemispheres (Fig. 7A). The developing central nervous system itself remains negative for MDP-1 throughout embryogenesis. As it has been shown for the developing ventral nerve cord (Sonnenfeld and Jacobs, 1995), hemocytes appear to be unable to penetrate the dense cephalic neuropile. In the posterior part of the embryo strong MDP-1 staining is especially noticeable along the lining of the developing gut, the Malpighian tubules and the posterior spiracles which represent the posterior external terminations of the larval tracheal system.

MDP-1 expression is not dependent on, but is modulated by embryonic apoptotic cell death

In addition to a role in the production of basement membrane components, *Drosophila* embryonic macrophages play a role in morphogenesis by phagocytosing extracellular debris, including apoptotic cells. We therefore examined whether the expression of MDP-1 may be correlated with the role of hemocytes in phagocytosis. Apoptosis begins in *Drosophila* embryos during late stage 11, and is most evident during head involution [stages 12-13] and CNS development [stages 13-15; (Abrams *et al.*, 1993)]. During embryogenesis, MDP-1 immunolabeling was heaviest in the procephalon during head involution (Fig. 8C) around the ventral nerve cord (Fig. 8E) and in the involutions of the midgut (Fig. 8E). A possible connection between MDP-1 expression and the onset of phagocytosis was assessed by examining 5H7 labeling in homozygous *Df(3R) H99* embryos which are deficient for apoptosis (White *et al.*, 1994). In homozygous *H99* embryos, the pattern of

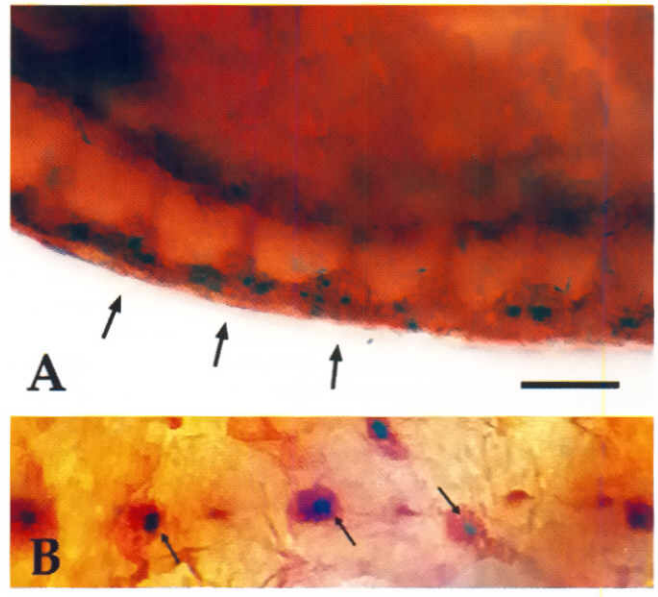

Fig. 5. The 5H7 mAb stains cells marked by a hemocyte-specific enhancer trap line. *Embryos were collected from the A109.1F2 enhancer trap stock and processed for β-galactosidase staining as described in the Methods section. After blue color development was visible in hemocyte nuclei, the embryos were incubated with 5H7 mAb for immunocytochemistry. Immunostaining is apparent in brown. Similar to Figure 4A the segmental reiteration of the midline pores is indicated by arrows in this sagittal view of the ventral nerve cord* **(A).** *The arrows in* **(B)** *indicate several MDP-1-positive cells along the ventral nerve cord which exhibit a dark blue β-galactosidase staining in their nuclei. Bar, (A) 30 μm and (B) 20 μm.*

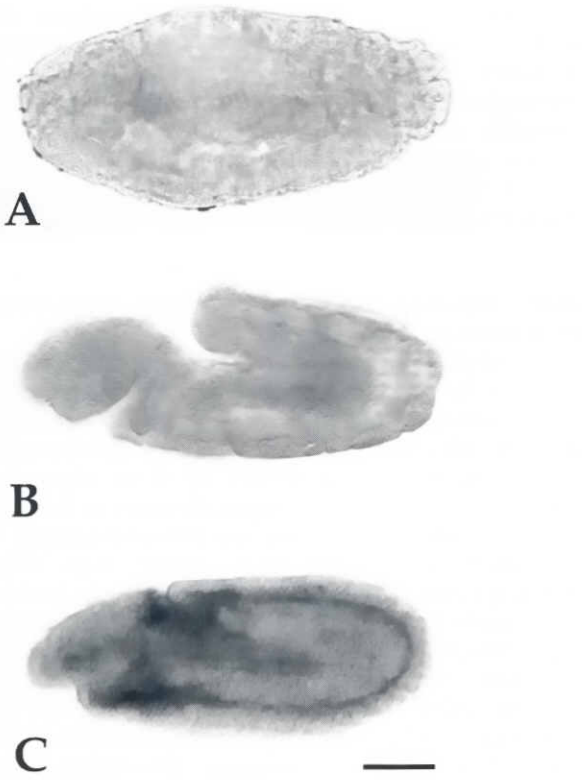

Fig. 6. MDP-1 is not detectable by 5H7 immunostaining in *Drosophila* embryos missing anterior or mesodermal tissues. *Embryos were collected from Bic-D^{IIIE48}/Bic-D^{7134} and sna^{IIG05}, twi^{S60}/CyO mutant Drosophila females, fixed and processed for 5H7 immunocytochemistry.* **(A)** shows a stage 13 embryo with a strong *Bic-D* phenotype, and **(B)** *a sna^{IIG05}, twi^{S60} double mutant embryo stained with 5H7 mAb.* **(C)** *shows a heterozygous sna^{IIG05}, twi^{S60}/CyO embryo (early embryonic stage 12) exhibiting normal MDP-1 staining. Bar, 100 μm.*

early immunolabeling of MDP-1 was identical to wildtype, although levels of expression were reduced in some tissues (Fig. 8B). During head involution, levels of MDP-1 labeling were significantly reduced in the procephalon, and slightly reduced around the Malpighian tubules, the posterior spiracles, and the hindgut (Fig. 8D). During late embryogenesis, MDP-1 immunolabeling close to wildtype levels was seen, however, the pattern of deposition was altered around the gut, reflecting perturbed gut morphogenesis in *H99* mutant embryos (Fig. 8E and F). Therefore normal apoptosis was not required for the induction of MDP-1 expression, however, MDP-1 deposition appeared to be reduced in amount and delayed in the absence of programmed cell death. This reduction of MDP-1 deposition correlated well with the different levels of apoptosis in these tissues, indicating that other genes within the *H99* deletion do not generally influence MDP-1 expression by a more direct mechanism.

Discussion

The data presented in this paper address some interesting questions concerning the differentiation of *Drosophila* hemocytes and the functional role these cells fulfill during embryogenesis. It had previously been established that hemocytes are derived from a small pool of anterior, mesodermal cells (Tepass *et al.*, 1994). From this anterior position they migrate throughout the embryo and begin to populate various organs, especially the surface of the central nervous system and the developing gut. Morphological evidence suggests that most, if not all, hemocytes will eventually differentiate into phagocytic macrophage-type cells. Hemocytes are often closely associated with areas of apoptotic cell death in the developing *Drosophila* embryo where they engage in the removal of cell debris (Abrams *et al.*, 1993; Tepass *et al.*, 1994; Sonnenfeld and Jacobs, 1995). Programed cell death in the *Drosophila* embryo commences at embryonic stage 11 and is initially prevalent in the developing nervous system (Abrams *et al.*, 1993). The finding that the number of hemocytes is unchanged in apoptosis-deficient embryos indicates that apoptotic events do not regulate the proliferation of hemocyte precursor cells. However, migratory hemocytes in these embryos do retain the flattened, fibroblast-like morphology of undifferentiated cells (Zhou *et al.*, 1995). These findings suggest that the final differentiation of hemocytes to phagocytic, macrophage-like cells only happens after these cells have started migrating and have left the anterior mesodermal hemocyte anlage. The differentiating tissues encountered by migrating, undifferentiated hemocytes may provide the necessary cues for final hemocyte differentiation to occur. In contrast to the early hemocyte marker protein Peroxidasin, MDP-1 is only expressed after hemocytes have left the procephalic mesoderm and have encountered areas where programed cell death is occurring. Although MDP-1 expression around the developing embryonic nervous system is greatly reduced in apoptosis-deficient embryos, MDP-1 is expressed near normal levels in other regions of the embryo, for example the gut, the posterior spiracles and the clypeolabrum. This indicates that programed cell death is not a «*sine qua non*» for MDP-1 expression to occur. However, MDP-1 expression correlates with further differentiation of hemocytes and the functional specialization for phagocytosis. One possibility is that the level of hemocyte phagocytic activity is dependent on the amount of cell debris encountered by the migrating hemocyte precursor cell. In *H99* embryos, the lack of apoptosis reduces the amount of cell debris encountered by macrophages, perhaps causing the lower levels of MDP-1 expression. MDP-1 will provide a useful marker in future studies addressing the mechanism of final hemocyte differentiation and how it is influenced by programed cell death.

Migrating hemocytes are the major source of extracellular matrix molecules in the *Drosophila* embryo. In addition to MDP-1, other extracellular matrix molecules which are secreted by these cells include Laminin, Tiggrin, Type IV Collagen, and Glutactin (Fessler *et al.*, 1994). All of these different ECM molecules have also been isolated from conditioned medium of *Drosophila* Kc cells. Campbell *et al.* (1987) isolated and biochemically characterized a proteoglycan-like molecule from Kc cells, they refer to as Papilin. Our experiments indicate that the 5H7 antigen might be the naturally occurring form of Kc cell Papilin. Not only do they have a very similar apparent molecular weight on SDS-PAGE gels and Kc cell Papilin can be specifically immunoprecipitated using the 5H7 mAb, but their overall amino acid composition is also very similar. In contrast, the carbohydrate composition of Papilin isolated from Kc cells differs significantly from that of 5H7 antigen preparations, which were isolated from *Drosophila* embryonic protein extracts. Campbell *et al.* (1987) reported a large variance in the glycosylation

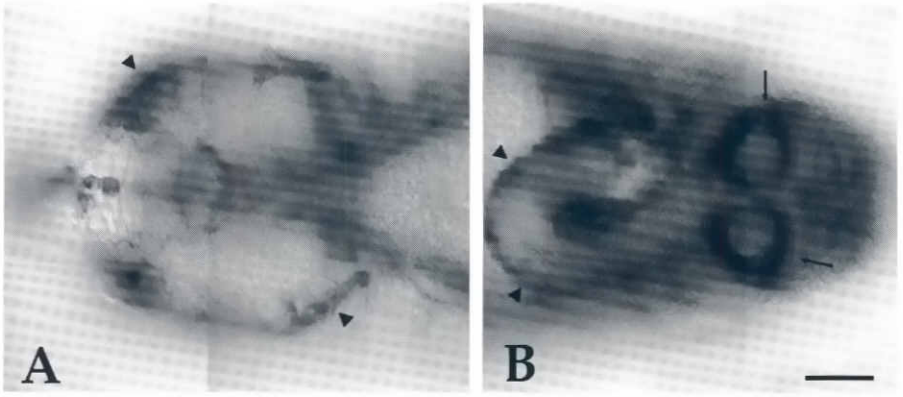

Fig. 7. MDP-1 is detected in areas of basement membrane deposition in late *Drosophila* embryos. *5H7 immunostaining in a late embryonic stage 13 Drosophila embryo. These two composite photographs show the anterior* **(A)** *and the posterior end* **(B)** *of the same embryo facing the dorsal side. Arrowheads in panel A indicate MDP-1 staining around the brain lobes and in panel B the basement membrane lining of the hindgut. The arrows in panel B point to the posterior spiracles which represent the terminal structures of the larval tracheal tree. Bar, 30 μm.*

content of Papilin isolated from different Kc cell lines and these differences are probably due to differences in the glycosylation patterns produced by different Kc cell lines and embryonic hemocytes.

Hemocytes appear to be responsible for the deposition of basement membranes surrounding many developing organs in the *Drosophila* embryo, such as the gut and the central nervous system. One specific acellular, extracellular membrane structure is the neural lamella, which unsheathes the embryonic central nervous system and constitutes a part of the insect 'blood-brain barrier' (Juang and Carlson, 1994). Our results indicate that MDP-1 is a component of this neural lamella, which provides an anionic domain and thereby may serve as a charge-barrier for cations in the hemolymph. Interestingly, Campbell *et al.* (1987) demonstrated that Papilin isolated from Kc cells is rich in acidic, negatively-charged carbohydrate side chains and in addition is heavily sulfated. MDP-1 might therefore contribute to the anionic charge barrier described by Juang and Carlson (1994).

As outlined in the introduction, proteoglycans are potent modulators of neuronal growth and axonal pathfinding by either providing permissive or inhibitory cues. Migratory hemocytes can be found in the hemolymph around the central nervous system, but appear to be unable to penetrate the dense neuropile. MDP-1 deposition is therefore restricted to the surface of the developing nerve cord and brain hemispheres. It is intriguing that deposition of MDP-1 surrounding the developing nerve cord starts at stage 11 shortly before axonogenesis commences during embryonic stage 12. It is therefore tempting to speculate that MDP-1 may contribute to an extracellular matrix boundary that delineates CNS structure and may provide a non-permissive barrier during axon pathfinding.

The importance of proteoglycans in axonal pathfinding in insects has been demonstrated by experiments performed by Wang and Denburg (1992). These authors showed that the addition of glycosaminoglycan side chains or of proteoglycan degrading enzymes perturbs the growth of pioneer axons and axon fasciculation in cultured cockroach embryos. It is evident from their experiments that proteoglycan-type molecules fulfill similar important roles during nervous system development in invertebrates as those which have been reported in vertebrate species. It will be informative to apply the genetic tools available in the *Drosophila* system to elucidate the developmental role of proteoglycans in embryonic development in general and that of MDP-1 in particular. The analysis of mutations in the gene for the MDP-1 core protein would enable us to test the suspected barrier functions of MDP-1 in the developing neural lamella in the *Drosophila* embryo.

Materials and Methods

Generation of 5H7 monoclonal antibody

Drosophila adherent embryonic cells were isolated and maintained in tissue culture on uncoated Falcon Primaria tissue culture plates as described by Patel *et al.* (1987). Adherent cells were collected after 2 days in culture and a crude membrane fraction was prepared according to Hortsch (1994) and used as an immunogen in BALB/c mice. Monoclonal antibodies producing hybrid cell lines were generated using standard techniques (Harlow and Lane, 1988) and tissue culture supernatants were screened using HRP-immunocytochemistry on whole-mount *Drosophila* embryos (Patel *et al.*, 1989). Positive cell lines were cloned using a soft agar cloning protocol (Bieber, 1994). Tissue culture supernatant from the 5H7 hybridoma cell line 5H7 (a monoclonal antibody that recognizes MDP-1, see below) was used for the immunocytochemistry experiments and 5H7 ascites fluid was generated in pristane-primed mice for the construction of 5H7 affinity matrices.

Immunoaffinity purification of MDP-1, immunoprecipitation procedure and SDS-polyacrylamide gel electrophoresis

The generation of affinity matrices using mAbs and the purification of polypeptides from total *Drosophila* embryonic protein extracts have been described in detail elsewhere (Hortsch, 1994). 200 ml of an total embryonic protein extract was passed over the 5H7-affinity column (5 ml) at 15 ml/h at 4°C. The eluted protein was neutralized with 1 M phosphate buffer, dialyzed against 0.1 M ammonium bicarbonate and concentrated by evaporation *in vacuum*. The same 5H7 mAb affinity matrix was also used for the immunoprecipitation experiments described in Figure 2. Purified MDP-1 and immunoprecipitated proteins were separated on 3 to 10% gradient SDS-polyacrylamide gels and stained using the silver stain method of Ansorge (1985).

Biochemical analysis of MDP-1

Protein determinations were performed according to Lowry *et al.* (1951). Bovine serum albumin was used as standard protein. Two independent MDP-1 preparations were analyzed in duplicates at 2 different protein concentrations.

Total neutral sugars were estimated by the phenol-sulphuric acid method of Dubois *et al.* (1956). To 100 μl of the sample was added 200 μl of a 5% phenol solution, followed by 800 μl of ice-cold concentrated sulphuric acid. Absorbance was read at 485 nm after 15 min and the carbohydrate content was calculated from a standard curve established using glucose.

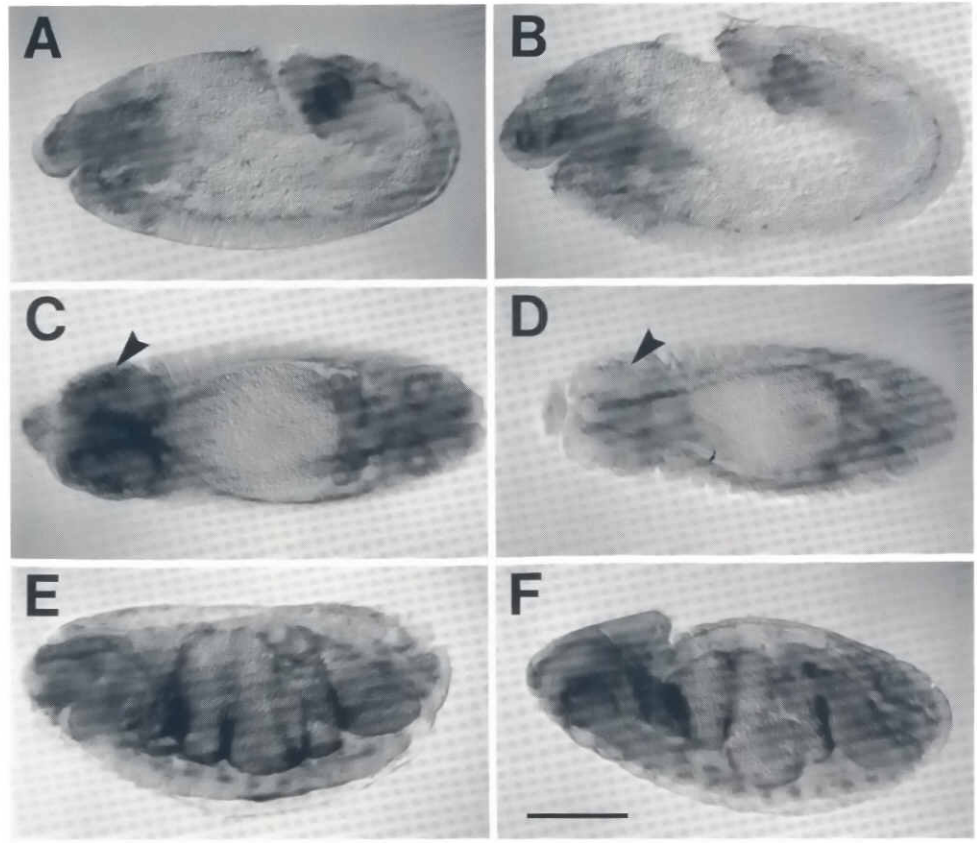

Fig. 8. Distribution of MDP-1 contrasted between wildtype and *H99* embryos. *5H7 mAb labeling in wildtype embryos* **(A, C, and E)** *and H99 embryos deficient for apoptosis* **(B, D, and F)** *is shown in sagittal view for late stage 11* **(A, B)** *and stage 16* **(E, F)** *and in dorsal view for early stage 13* **(C, D).** *5H7 labeling is similar in wildtype and H99 embryos around the stomodeum, gut and posterior spiracles. However, labeling around the brain in wildtype, during and after head involution (arrowhead in C) is missing in head involution-defective H99 embryos (D, arrowhead). Anterior is left in all panels. Bar, 200 μm.*

Uronic acids were assayed by the carbazole reaction of Bitter and Muir (1962). 0.5 ml of 0.025 M sodium tetraborate in concentrated sulphuric acid was cooled to -70°C and layered over 0.1 ml of sample or glucuronolactone standard. Closed tubes were shaken vigorously, heated for 10 min at 100°C, and finally cooled to room temperature. After addition of 20 μl of carbazole reagent (0.125% carbazole in methanol) the samples were vortexed, boiled at 100°C for 15 min, and cooled to room temperature. The absorbance was read at 530 nm.

A modified Elson-Morgan reaction according to Augustyniak and Augustyniak (1966) was used to estimate amino sugars. Samples containing 1-20 mg of amino sugars in a volume of 0.3 ml were mixed with an equal volume of 4 N HCl in Pyrex screw-capped tubes and digested at 100°C for 10 to 12 h. The samples were neutralized with 0.4 ml of 2 M sodium carbonate and gently agitated before 0.5 ml of 2% acetyl acetone in 1.5 M sodium carbonate were added. The tubes were capped tightly and heated in a boiling water bath for 20 min. After cooling to room temperature 1 ml of ethanol was added followed by 0.5 ml of Ehrlich's reagent. The tubes were shaken vigorously to expel excess carbon dioxide and absorbance was read at 530 nm. Glucosamine was used for the standard.

Drosophila stocks

Wild type embryos were collected from an Oregon R stock. The A*109.1F2* line is a hemocyte-specific enhancer trap line with a P-element insert near the collagen type IV gene on the 2nd chromosome (Wilson *et al.*, 1989). This line was kindly provided by D. Kimbrell (Baylor College). *Bic-D*^{IIIE48}*/CyO* and *Bic-D*^{7134}*/CyO* flies were from B. Suter (McGill University). To obtain a high frequency of B*ic-D* mutant embryos, transheterozygous females (*Bic-D*^{IIIE48}*/Bic-D*^{7134}) were kept at 18°C for embryo collections. The *sna*^{IIG05}, *twi*^{S60}*/CyO* double mutant line was provided by R. Bodmer (University of Michigan). Embryos deficient for programmed cell death were obtained from D*f(3R)H99/TM6[Ubx-lacZ]* flies, which lack *hid* and *rpr* function (White *et al.*, 1994). The D*f(3R)H99* stock was provided by H. Steller (MIT). *Df(3R)H99* homozygous embryos were identified and selected for by their negative β-galactosidase staining. Embryos were staged according to Campos-Ortega and Hartenstein (1985).

Whole-mount immunocytochemistry of Drosophila embryos

Immunolocalization in whole-mount embryos was performed using HRP-conjugated goat anti-mouse IgG secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) as outlined by Patel *et al.* (1989). β-galactosidase localization was performed as described by Klämbt *et al.* (1991) with the following modifications. Embryos were fixed for 15 min with PBS plus 4% formaldehyde and devitellinized using ethanol instead of methanol. The embryos were incubated in the staining solution using X-gal as the substrate at 37°C for up to 3 days. Subsequently the embryos were processed for immunocytochemistry using the 5H7 mAb. Stained embryos were mounted in PBS with 70% glycerol and observed and photographed with a Nikon Optiphot microscope using either phase contrast or Nomarski optics.

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References

ABRAMS, J. M., WHITE, K., FESSLER, L. I. and STELLER, H. (1993). Programmed cell death during *Drosophila* embryogenesis. *Development 117:* 29-43.

ANSORGE, W. (1985). Fast and sensitive detection of protein and DNA bands by treatment with potassium permanganate. *J. Biochem. Biophys. Methods 11:* 13-20.

AUGUSTYNIAK, H. AND AUGUSTYNIAK, J. (1966). Anhydride-type basic artifacts of amino acids. *Anal. Biochem. 15:* 163-167.

BIEBER, A. J. (1994). Analysis of cellular adhesion in cultured cells. In *Drosophila melanogaster: Practical Uses in Cell Biology* (Eds. L. Goldstein and E. Fyrberg). Methods in Cell Biology Vol. 44 Academic Press, San Diego, pp. 683-696.

BITTER, T. and MUIR, H. M. (1962). A modified uronic acid carbazole reaction. *Anal. Biochem. 4:* 330-334.

BREHELIN, M. (1982). Comparative study of structure and function of blood cells from two *Drosophila* species. *Cell Tissue Res. 221:* 607-615.

BROWER, D. L., PIOVANT, M., SALATINO, R., BRAILEY, J. and HENDRIX, M. J. C. (1987). Identification of a specialized extracellular matrix component in *Drosophila* imaginal discs. *Dev. Biol. 119:* 373-381.

CAMPBELL, A. G., FESSLER, L. I., SALO, T. and FESSLER, J. H. (1987). Papilin: a *Drosophila* proteoglycan-like sulfated glycoprotein from basement membranes. *J. Biol. Chem. 262:* 17605-17612.

CAMPOS-ORTEGA, J. A. and HARTENSTEIN, V. (Eds.) (1985). *The Embryonic Development of Drosophila melanogaster.* Springer-Verlag, Berlin, Heidelberg, New York, Tokyo.

CASSARO, C. M. and DIETRICH, C. P. (1977). Distribution of sulfated mucopolysaccharides in invertebrates. *J. Biol. Chem. 252:* 2254-2261.

COLE, G. J., LOEWY, A. and GLASER, L. (1986). Neuronal cell-cell adhesion depends on interactions of N-CAM with heparin-like molecules. *Nature 320:* 445-447.

CROSS, D. P. and SANG, J. H. (1978). Cell culture of individual *Drosophila* embryos. I. Development of wild-type cultures. *J. Embryol. Exp. Morphol. 45:* 161-172.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. and SMITH, F. (1956). Colorimetric method for determination of sugar and related substances. *Anal. Chem. 28:* 350-356.

EDWARDS, J. S., SWALES, L. S. and BATE, M. (1993). The differentiation between neuroglia and connective tissue sheath in insect ganglia revisited: the neural lamella and perineurial sheath cells are absent in a mesodermless mutant of *Drosophila. J. Comp. Neurol. 333:* 301-308.

FAISSNER, A., CLEMENT, A., LOCHTER, A., STREIT, A., MANDL, C. and SCHACHNER, M. (1994). Isolation of a neural chondroitin sulfate proteoglycan with neurite outgrowth promoting properties. *J. Cell Biol. 126:* 783-799.

FESSLER, L. I., NELSON, R. E. and FESSLER, J. H. (1994). *Drosophila* extracellular matrix. *Methods Enzymol. 245:* 271-294.

FRIEDLANDER, D. R., MILEV, P., KARTHIKEYAN, L., MARGOLIS, R. K., MARGOLIS, R. U. and GRUMET, M. (1994). The neuronal chondroitin sulfate proteoglycan neurocan binds to the neural cell adhesion molecules Ng-CAM/L1/NILE and N-CAM, and inhibits neuronal adhesion and neurite outgrowth. *J. Cell Biol. 125:* 669-680.

GRANER, M., STUPKA, K. and KARR, T. L. (1994). Biochemical and cytological characterization of DROP-1: a widely distributed proteoglycan in *Drosophila. Insect Biochem. Mol. Biol. 24:* 557-567.

HARLOW, E. and LANE, D. (Eds.) (1988). *Antibodies - A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

HOFFMAN, S. and EDELMAN, G. M. (1987). A proteoglycan with HNK-1 antigenic determinants is a neuron-associated ligand for cytotactin. *Proc. Natl. Acad. Sci. USA 84:* 2523-2527.

HÖGLUND, L. (1976). The comparative biochemistry of invertebrate mucopolysaccharides-V. Insecta (*Calliphora erythrocephala). Comp. Biochem. Physiol. 53B:* 9-14.

HORTSCH, M. (1994). Preparation and analysis of membranes and membrane proteins from *Drosophila.* In *Drosophila melanogaster: Practical Uses in Cell Biology* (Eds. L. Goldstein and E. Fyrberg). Methods in Cell Biology Vol. 44 Academic Press, San Diego, pp. 289-301.

HORTSCH, M., BIEBER, A. J., PATEL, N. H. and GOODMAN, C. S. (1990a). Differential splicing generates a nervous system-specific form of *Drosophila* neuroglian. *Neuron 4:* 697-709.

HORTSCH, M., PATEL, N. H., BIEBER, A. J., TRAQUINA, Z. R. and GOODMAN, C. S. (1990b). *Drosophila* neurotactin, a surface glycoprotein with homology to serine esterases, is dynamically expressed during embryogenesis. *Development 110:* 1327-1340.

JUANG, J.-L. and CARLSON, S. D. (1994). Analog of vertebrate anionic sites in blood-brain interface of larval *Drosophila. Cell Tissue Res. 277:* 87-95.

KJELLEN, L. and LINDAHL, U. (1991). Proteoglycans: structures and interactions. *Annu. Rev. Biochem. 60:* 443-475.

KLÄMBT, C., JACOBS, J. R. and GOODMAN, C. S. (1991). The midline of the *Drosophila* central nervous system: a model for the genetic analysis of cell fate, cell migration, and growth cone guidance. *Cell 64:* 801-815.

LANDER, A. D. (1993). Proteoglycans in the nervous system. *Curr. Opin. Neurobiol. 3:* 716-723.

LANDER, A. D., FUJII, D. K., GOSPODAROWICZ, D. and REICHARDT, L. F. (1982). Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. *J. Cell Biol. 94:* 574-585.

LANDER, A. D., FUJII, D. K. and REICHARDT, L. F. (1985). Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. *J. Cell Biol. 101:* 898-913.

LETOURNEAU, P. C., CONDIC, M. L. and SNOW, D. M. (1994). Interactions of developing neurons with the extracellular matrix. *J. Neurosci. 14:* 915-928.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem. 193:* 265-275.

MARGOLIS, R. K. and MARGOLIS, R. U. (1994). Nervous tissue proteoglycans. In *Proteoglycans* (Ed. P. Jolles). Vol. 70. Birkhäuser Verlag, Basel, pp. 145-177.

MATTHEW, W. D., GREENSPAN, R. J., LANDER, A. D. and REICHARDT, L. F. (1985). Immunopurification and characterization of a neuronal heparan sulfate proteoglycan. *J. Neurosci. 5:* 1842-1850.

MAUREL, P., RAUCH, U., FLAD, M., MARGOLIS, R. K. and MARGOLIS, R. U. (1994). Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA 91:* 2512-2516.

NAKATO, H., FUTCH, T. A. and SELLECK, S. B. (1995). The *division abnormally delayed (dally)* gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila. Development 121:* 3687-3702.

NELSON, R. E., FESSLER, L. I., TAKAGI, Y., BLUMBERG, B., KEENE, D. R., OLSON, P. F., PARKER, C. G. and FESSLER, J. H. (1994). Peroxidasin: a novel enzyme-matrix protein of *Drosophila* development. *EMBO J. 13:* 3438-3447.

OLDBERG, A. and RUOSLAHTI, E. (1982). Interactions between chondroitin sulfate proteoglycan, fibronectin, and collagen. *J. Biol. Chem. 257:* 4859-4863.

OOHIRA, A., MATSUI, F. and KATOH-SEMBA, R. (1991). Inhibitory effects of brain chondoitin sulfate proteoglycans on neurite outgrowth from PC12D cells. *J. Neurosci. 11:* 822-827.

PATEL, N. H., MARTIN-BLANCO, E., COLEMAN, K. G., POOLE, S. J., ELLIS, M. C., KORNBERG, T. B. and GOODMAN, C. S. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell 58:* 955-968.

PATEL, N. H., SNOW, P. M. and GOODMAN, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in *Drosophila. Cell 48:* 975-988.

RUOSLAHTI, E. (1988). Structure and biology of proteoglycans. *Annu. Rev. Cell Biol. 4:* 229-255.

SAUNDERS, S. and BERNFIELD, M. (1988). Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. *J. Cell Biol. 106:* 423-430.

SNOW, D. M., LEMMON, V., CARRINO, D. A., CAPLAN, A. I. and SILVER, J. (1990). Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro. Exp. Neurol. 109:* 111-130.

SONNENFELD, M. J. and JACOBS, J. R. (1995). Macrophages and glia participate in the removal of apoptotic neurons from the *Drosophila* embryonic nervous system. *J. Comp. Neurol. 359:* 644-652.

SPRING, J., PAINE-SAUNDERS, S. E., HYNES, R. O. and BERNFIELD, M. (1994). *Drosophila* syndecan: conservation of a cell-surface heparan sulfate proteoglycan. *Proc. Natl. Acad. Sci. USA 91:* 3334-3338.

TEPASS, U., FESSLER, L. I., AZIZ, A. and HARTENSTEIN, V. (1994). Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. *Development 120:* 1829-1837.

WANG, L. and DENBURG, J. L. (1992). A role for proteoglycans in the guidance of a subset of pioneer axons in cultured embryos of the cockroach. *Neuron 8:* 701-714.

WHITE, K., GRETHER, M. E., ABRAMS, J. M., YOUNG, L., FARRELL, K. and STELLER, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Science 264:* 677-683.

WILSON, C., PEARSON, R. K., BELLEN, H. J., O'KANE, C. J., GROSSNIKLAUS, U. and GEHRING, W. J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Dev. 3:* 1301-1313.

ZHOU, L., HASHIMI, H., SCHWARTZ, L. M. and NAMBU, J. R. (1995). Programmed cell death in the *Drosophila* central nervous system midline. *Curr. Biol. 5:* 784-790.

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