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Isolation from Chick Somites of a Glycoprotein Fraction That Causes Collapse of Dorsal Root Ganglion Growth Cones

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Summary

The segmented pattern of peripheral spinal nerves in higher vertebrates is generated by interactions between nerve cells and somites. Neural crest cells, motor axons, and sensory axons grow exclusively through anterior-half sclerotome. In chick embryos, posterior cells bind the lectins peanut agglutinin (PNA) and Jacalin. When liposomes containing somite extracts are applied to cultures of chick sensory neurons, growth cones collapse abruptly, recovering within 4 hr of liposome removal. Collapse activity is eliminated by immobilized PNA, and SDS-PAGE demonstrates two major components (48K and 55K), which are absent from anterior-half sclerotome. Rabbit polyclonal antibodies against these components recognize only posterior cells and may also be used to eliminate collapse activity. We suggest that spinal nerve segmentation is produced by inhibitory interactions between these components and growth cones.

Introduction

The search for molecules that guide growing axons during vertebrate development requires the study of simple and accessible systems. One such system, common to all higher vertebrate embryos, involves the generation of a segmented pattern of peripheral spinal nerves. Spinal nerve segmentation is known to be orchestrated by segmentation in the paraxial (somite) mesoderm alongside the neural tube (Lehmann, 1927; Detwiler, 1934), and in higher vertebrate embryos it is further determined by the subdivision of each somite into anterior (A, cranial) and posterior (P, caudal) halves (Keynes and Stern, 1984, 1988; Tosney, 1988a).

Somites first appear as epithelial rosettes and then undergo a rapid morphogenetic rearrangement to form three major subdivisions: the dermatome (presumptive dermis), myotome (presumptive skeletal muscle), and sclerotome (presumptive vertebral column). When migrating neural crest cells leave the neural tube and encounter the sclerotomes, they are confined exclusively to each anterior-half sclerotome, entering there as soon as it dissociates from the epithelial somite (Rickmann et al., 1985; Bronner-Fraser, 1986; Teillet et al., 1987; Loring and Erickson, 1986; Erickson et al., 1989). The crest-derived dorsal root ganglia (DRG) subsequently form in the anterior-half sclerotomes, immediately adjacent to the neural tube (Keynes and Stern, 1985; Teillet et al., 1987; Lallier and Bronner-Fraser, 1988; Kalcheim and Teillet, 1989). At each segmental level, several hours after the first crest cells entered the sclerotome, motor axons grow out from the ventral neural tube and sensory axons sprout from the ganglion, again exclusively within the anterior-half sclerotome (Keynes and Stern, 1984). A-P reversal of a short strip of presumptive somite mesoderm forces axons to traverse the posterior (original anterior)-half of each reversed segment (Keynes and Stern, 1984).

In principle, the preference of nerve cells for growth through anterior-half sclerotome could result from the operation of adhesive/attractive influences in the anterior half, inhibitory/repulsive influences in the posterior half, or a combination of the two. Prior ablation of the somites (Lewis et al., 1981) or sclerotomes (Tosney, 1988b) abolishes the segmented pattern of axon outgrowth; motor axons then grow out evenly along the length of the neural tube, as they do from neural tubes isolated in vitro (Burrows, 1911). This observation shows that axon growth can take place in the absence of anterior-half sclerotome and suggests that the dominant influence causing neural segmentation may be inhibitory, residing in the posterior-half sclerotome.

The fact that no known cell or substrate adhesion molecule has been shown to play any critical role in peripheral nerve segmentation is at least consistent with this view. Immunohistochemical studies using monoclonal antibodies to N-CAM, N-cadherin, laminin, and fibronectin have failed to demonstrate any differential distribution of these molecules within the sclerotome (Rickmann et al., 1985; Krotoski et al., 1986; Duband et al., 1987; Hatta et al., 1987; Mackie et al., 1988). The substrate adhesion molecule cytotactin/tenascin/J1 has been reported to be concentrated in anterior-half sclerotome (Tan et al., 1987; Mackie et al., 1988), but a more recent study has revealed that its distribution is more complex, both spatially and temporally, than first suspected; although it may modulate the growth of crest cells within the sclerotome, it is unlikely to play a key role in determining their preference for anterior-half sclerotome (Stern et al., 1989).

Histochemical studies using a variety of plant lectins have revealed a difference between anterior-half and posterior-half sclerotome of potential importance for the inhibitory hypothesis: peanut agglutinin (PNA) binds only the posterior (axon-repelling)-half sclerotome (Stern et al., 1986). In this paper we describe the

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isolation of PNA binding glycoproteins from the somites of chick embryos and show that components present in this material are likely to be responsible for inhibition of growth cone advance.

Results

Lectin Histochemistry

The spatial and temporal distribution of somite PNA receptors was studied using lectin histochemistry to determine whether their expression patterns are compatible with a possible role in axon guidance. The results of FITC-PNA staining of frozen sections of chick embryos, containing somites that have differentiated into dermomyotome and sclerotome, are shown in Figure 1a. As described previously by Stern et al. (1986), who used a PNA-HRP conjugate at stage 16, FITC-PNA is seen to bind only to cells of the posterior-half sclerotome. Binding is inhibited completely by competing sugar, 0.2 M lactose, a ligand for PNA (Lotan et al., 1975). No fluorescence is visible in the early epithelial somites, and labeling appears several hours after they have undergone their morphogenetic rearrangement into dermomyotome and sclerotome. During the somite stages of development in any particular embryo, the youngest somite to show clearly detectable PNA binding is placed 10 somites anterior to that which has most recently segmented; this position is also approximately 7 somites anterior to the oldest epithelial somite. From the earliest stage of visible staining in the somite, fluorescence is confined to the posterior-half sclerotome cells (P-cells). Subsequently, P-cells continue to show binding until the arrangement of sclerotomes is lost, during the development of the definitive vertebral column.

The binding of PNA to its ligand, Gal-β(1-3)-GalNAc, is prevented by terminal sialylation of this disaccharide moiety (Lotan et al., 1975). Another lectin, Jacalin, also binds to these residues (Sastry et al., 1986), but is reported to be insensitive to their terminal sialylation. Detailed studies on Jacalin (Hagiwara et al., 1988), however, reveal apparent variability of both structure and binding affinities of Jacalin from different sources. To confirm that the source of lectin used here does indeed recognize sialylated residues, dot-blots of fetuin and asialofetuin were stained with HRP conjugates of PNA and Jacalin: PNA bound only to asialofetuin, whereas Jacalin was found to recognize both glycoconjugates. When FITC-Jacalin was applied to frozen sections of chick embryos, it behaved exactly as PNA in the somites (Figure 1b), showing that the observed pattern of lectin staining is not due to differential sialylation in the two sclerotome halves. Isolated P-cells stained with FITC-PNA are shown in Figure 1c; they bound the lectin at their surfaces, and over the course of about 30 min the initial ring reaction evolved into patches, consistent with a cell surface location for PNA binding material. Isolated A-cells did not bind the lectin.

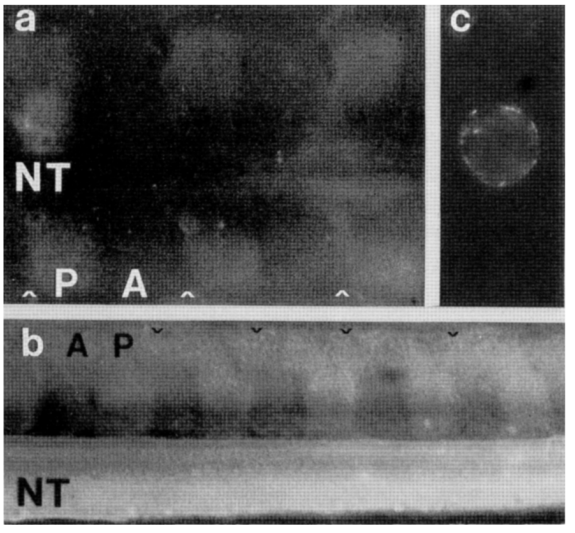

Figure 1. Lectin Staining of Chick Embryo Somites

(a) Longitudinal frozen section of a stage 17 embryo stained with 20 μg/ml FITC-PNA at 37°C for 4 hr. Staining is confined to the posterior half (P) of each sclerotome and is also seen in the neural tube (NT); the anterior-half sclerotome is marked (A), as are the segment boundaries (arrowheads). Magnification 80×.

(b) Sagittal frozen section of a stage 17 embryo stained with 20 μg/ml FITC-Jacalin at 37°C for 4 hr. Staining in sclerotomes is confined to the posterior halves (P), with appreciable staining in the neural tube (NT). (A) anterior-half sclerotome; arrowheads denote the segment boundaries. Magnification 80×.

(c) Isolated cell from dissociated posterior-half sclerotome, stained with 20 μg/ml FITC-PNA at 0°C for 30 min. Staining is confined to the cell periphery. Magnification 500×.

Metabolic Radiolabeling of Somite Cells

To facilitate the detection of glycoconjugates during lectin affinity chromatography, somites were labeled metabolically with [^{3}H]galactose. The accumulation of radiolabeled, phosphotungstic acid-precipitable material by somite cells incubated in medium containing [^{3}H]galactose is shown in Figure 2a. The net incorporation increased slowly for the first hour; the rate of increase then rose, became maximal after approximately 3 hr, and subsequently fell. After 6 hr, however, sufficient radioactivity for detection of material by affinity chromatography was achieved, 80% of this material being recoverable in the detergent-solubilized fraction. Routinely, experiments beyond this time point were not attempted because of the need to avoid substantial embryonic development in culture.

PNA Affinity Chromatography of [^{3}H]Galactose-Labeled Somite Material

The result of a typical elution profile obtained when radiolabeled somite material was fractionated on immobilized PNA is shown in Figure 2b. Nonbinding material was recovered as a large, broad peak of radioactivity by washing the column with buffer. Subsequent elution with 0.2 M lactose resulted in a single narrow peak of radioactivity, representing glycoconjugates

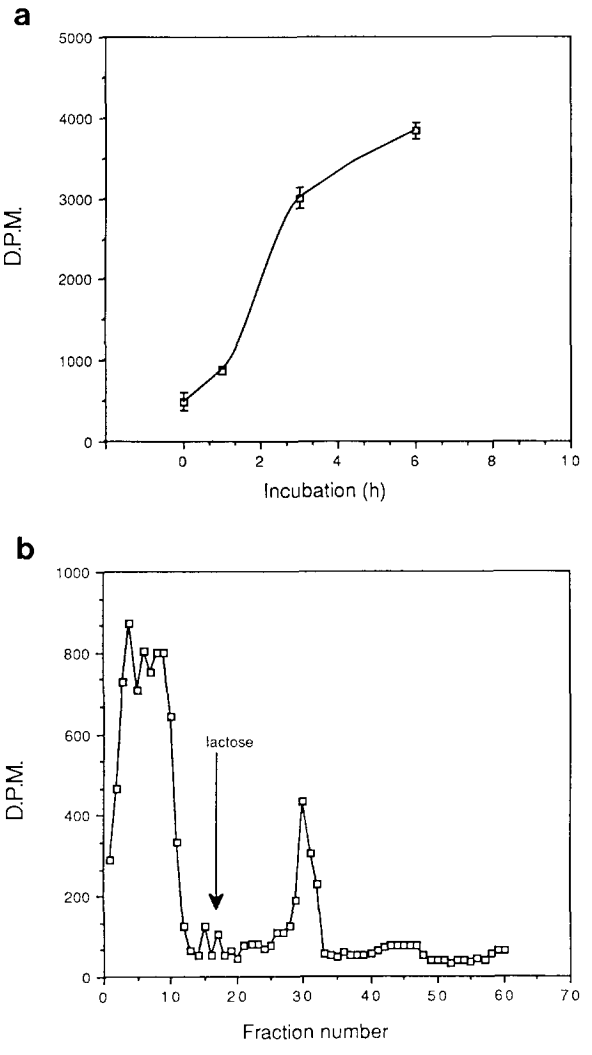

Figure 2. Affinity Chromatography of Somites Labeled Metabolically with [^{3}H]Galactose

(a) Time course of labeling of somite strips. Strips from 15 stage 17–19 embryos were labeled with 2 μCi of [^{3}H]galactose in DMEM for each time point. Labeled strips were washed with PBS, homogenized in 2% (w/v) BSA in PBS, and precipitated with 1% (w/v) phosphotungstic acid in 0.5 M HCl. Washed precipitates were solubilized in 1 M NaOH for scintillation counting in Ecoscint. Each time point represents the mean of five separate experiments ± SEM.

(b) Somite strips from 50 stage 17–19 embryos were labeled metabolically with [^{3}H]galactose for 8 hr, solubilized in TBB, and passed through a column of 1 ml of immobilized PNA. The elution profile shows a large peak of radioactivity that does not bind to the column, and elution with 0.2 M lactose (arrow) results in a single peak of radioactivity with a maximum at fraction 30.

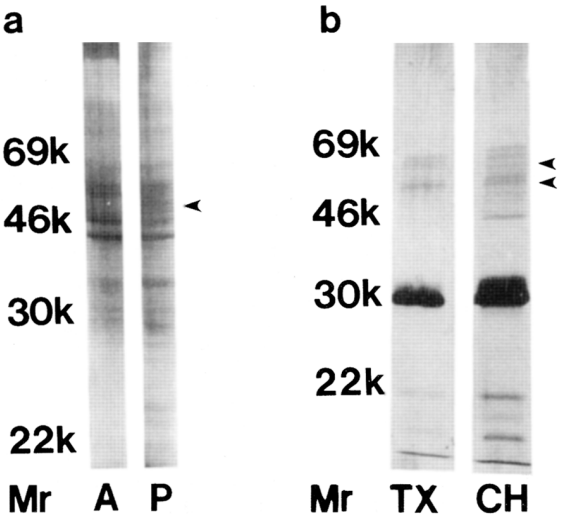

Figure 3. SDS-PAGE Analysis of Separated Half-Sclerotomes and PNA Binding Glycoproteins from Detergent-Solubilized Somite Strips

(a) The sclerotomes of 20 stage 17–19 embryos were dissected manually into anterior (A) and posterior (P) halves. Material analyzed on a 6% gel and stained with silver is shown. It should be noted that components of apparent M_r 48K and 55K (arrowhead) are absent from the anterior half. The positions of molecular weight markers are shown.

(b) Somite strips from 60 stage 17–19 embryos were solubilized in either TBB (TX) or 2% (w/v) CHAPS in PBS (CH); both extraction solutions were made 1 mM with respect to $CaCl_2$ and $MnCl_2$. The figure shows a comparison by SDS-PAGE on a 7.5% gel; TBB-solubilized material binds to a PNA-agarose column (see Figure 2b), and CHAPS-solubilized material binds to PNA-agarose as used in the collapse assay. The gel is stained with silver. In both TBB and CH samples the washed beads have been eluted with SDS-PAGE denaturing buffer at 100°C for 5 min, hence the presence of sizable quantities of PNA subunits at 30K. Bands of apparent M_r <30K are also found when fresh PNA-agarose is eluted with SDS-PAGE denaturing buffer in control experiments. It should be noted that components of apparent M_r 48K and 55K (arrowheads) are present in both TBB- and CH-extracted material.

that bind to PNA. This profile is similar to that reported in other studies using PNA affinity chromatography (e.g., Carter and Sharon, 1975). SDS-PAGE analysis, under reducing conditions, of the lactose-eluted peak is shown in Figure 3b. Major silver staining bands of apparent M_r 48K, 55K, and 60K were visualized in Triton X-100-solubilized material, and additional bands were seen when CHAPS was used. Two of the major components, at 48K and 55K, correspond to those present on one-dimensional gels of material obtained from dissection of posterior-half sclerotomes and were not present in gels of material from anterior-half sclerotomes (Figure 3a).

Growth of Axons on Substrates Coated with PNA Binding Material from Somites

A simple bioassay to detect inhibition of neurite extension was devised. This involved measuring axon elongation from DRG cultured on substrates to which isolated materials had been attached.

A comparison of the extent of axon elongation on substrates coated with bovine serum albumin (BSA) alone, or BSA combined with the glycoprotein fraction eluted from the PNA column, is shown in Figure 4. The median extent of outgrowth on the former sub-

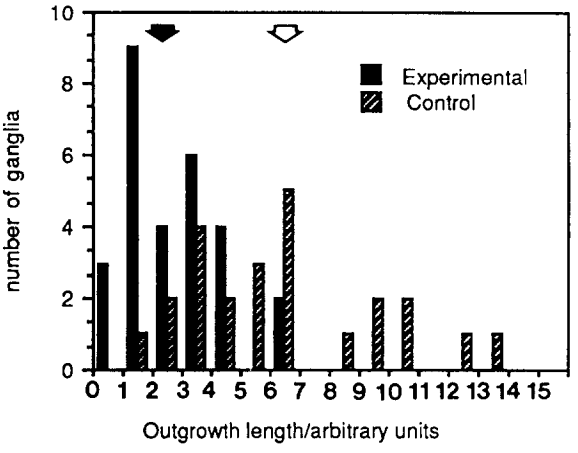

Figure 4. Substrate Bioassay for Inhibition of Neurite Growth

Isolated DRG from stage 33-35 embryos were divided into two approximately equal parts and placed on 1 cm squares of Hybond N-treated with either 0.1% BSA in TBB (control; cross-hatched) or glycoproteins isolated by PNA affinity chromatography (experimental; shaded). Neurite outgrowth was measured in arbitrary units using an eyepiece graticule. Median outgrowth is lower on the glycoprotein-treated substrate (black arrow) than on the BSA-treated substrate (open arrow).

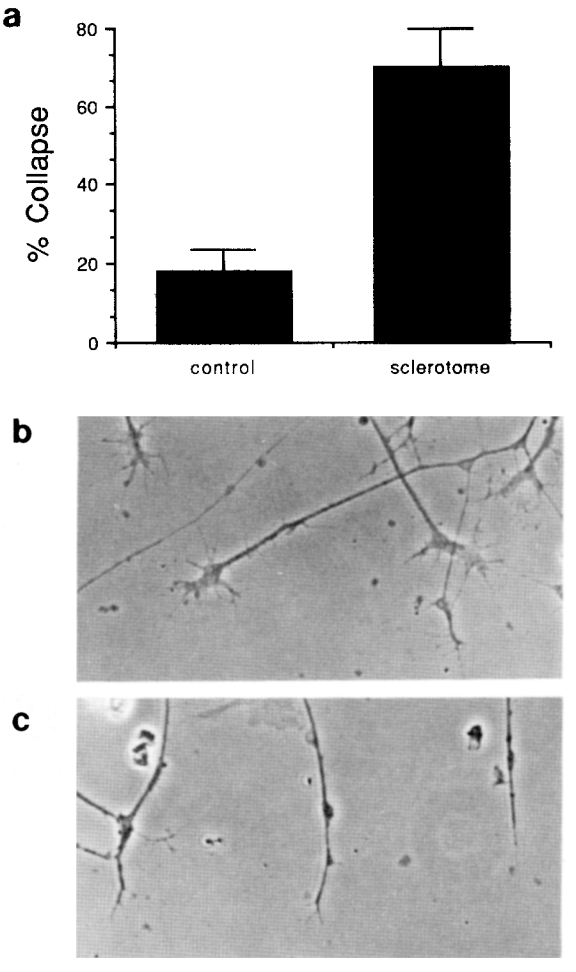

Figure 5. Growth Cone Collapse Activity in Chick Embryo Sclerotome

(a) DRG cultures treated with liposomes containing CHAPS-extracted sclerotome show a marked increase in the percentage of growth cones with a collapsed morphology when compared with those treated with plain liposomes (control). A representative experiment is shown, and the error bars represent 95% confidence limits based on sampling error. The same results are obtained when a CHAPS extract of whole stage 17-19 embryo trunks is used. A paired t-test analysis of replicate experiments shows significant differences at $P < 10^{-6}$ ($n = 7$).

(b) A phase-contrast micrograph of DRG growth cones on a laminin substrate showing characteristic spread morphology despite treatment with control liposomes. Because each growth cone possesses some lamellipodia and/or filopodia, each is scored as "spread." Magnification 500×.

(c) A phase-contrast micrograph of collapsed growth cones 1 hr after the application of liposomes incorporating a CHAPS extract of stage 17-19 chick embryo trunks. Magnification 500×.

strate is 6.0 arbitrary units (95% confidence limits 4.0-8.5), whereas on the latter it is 2.0 units (95% confidence limits 1.0-3.26); 1 arbitrary unit is approximately equal to 0.4 mm. Although axons were able to extend on both substrates, addition of the PNA binding material substantially reduced extension.

Growth Cone Collapse Assay

The collapse assay developed by Raper and Kapfhammer (1990) measures the ability of detergent-solubilized tissue fractions, incorporated into the bilayer of liposomes, to cause retraction of growth cones extending in vitro. This behavior mimics that seen when CNS growth cones meet PNS axons and vice versa (Kapfhammer and Raper, 1987a, 1987b). In the assay, liposomes are formed by removal of detergent from a mixture of defined phospholipids (phosphatidyl choline and phosphatidyl serine) and solubilized tissue. To make a size comparison between these liposomes and growth cones, freeze-fracture replicas of liposomes incorporating material from a homogenate of chick embryo trunks were examined. Liposomes had an elliptical profile, with a mean maximum diameter of 0.35 μm (SE = 0.12 μm) and a mean minimum diameter of 0.24 μm (SE = 0.11 μm; n = 166).

The results of treating DRG axons (growing on laminin) with liposomes containing sclerotome-derived proteins are shown in Figure 5a. The average number of growth cones in a collapsed (Figure 5c) rather than spread (Figure 5b) state rose markedly, from 17% (±6%) to 72% (±11%). Normal morphology was regained within 4 hr of replacement with liposome-free culture medium. This degree of collapse was achieved with a standard quantity of 220 μg of protein per culture well. Larger amounts of protein (up to 1.8 mg per well) failed to elicit more than 70%-80% collapse; lower quantities (110 μg) produced only 48% collapse. Growth cones derived from four separate neural tube explants, however, showed 100% (SE = 5%) collapse when standard quantities of protein were used, as

compared with control values of 16% (SE = 10%) when plain liposomes were used. The leading edges of other cells in the cultures (fibroblasts and glial cells) did not appear to be affected when examined at the standard interval of 1 hr after liposome addition.

To determine whether the PNA binding molecules from the somites are responsible for growth cone collapse, detergent-solubilized trunk extracts were first adsorbed with PNA immobilized on Sepharose 4B beads and liposomes were made with the remaining material. Figure 6 shows that this treatment eliminated all collapsing activity from the extract. This activity, moreover, could be recovered from the beads by eluting them with 0.4 M lactose. As a control, trunk extracts were treated with unconjugated Sepharose 4B beads; this treatment failed to remove any collapsing activity from the extract.

These results strongly suggest that at least some of the PNA binding molecules of the somite are capable of causing growth cone collapse. SDS-PAGE analysis of the glycoproteins bound to the immobilized lectin is shown in Figure 3b. It may be noted that the components of apparent M_r 48K and 55K are present. Furthermore, three separate determinations of uptake, using radiolabeled PNA binding glycoproteins isolated from somites whose polypeptides had been labeled with [^{35}S]methionine, showed that 85% (±10%) of the radioactivity was incorporated into the liposomes.

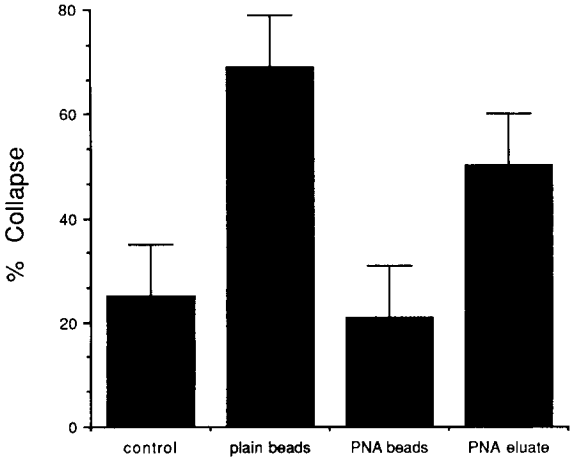

Figure 6. Growth Cone Collapse Activity Resides in PNA Binding Molecules

Liposomes incorporating stage 17–19 chick embryo trunk proteins that have been incubated at 4°C with unconjugated Sepharose 4B beads cause a marked increase in the percentage of collapsed growth cones (70% ± 9%; plain beads), compared with protein-free liposomes (23% ± 9%; control). Incubation of the trunk proteins with immobilized PNA at 4°C (PNA beads) results in reduction of collapse to control levels (18% ± 10%). Elution of PNA binding glycoproteins from the immobilized lectin, with 0.4 M lactose at 4°C, results in recovery of a substantial fraction of collapse (50% ± 10%; PNA eluate). The figure summarizes the results of a typical experiment. Analysis of replicates using a paired t-test shows significant differences between treatment with plain beads and PNA beads, and PNA eluate and control at $P = 2.4 \times 10^{-3}$ ($n = 5$) and $P = 1.5 \times 10^{-2}$ ($n = 4$), respectively.

Affinity Chromatography Using Immobilized Immune IgG

Treatment of somite extracts with immobilized rabbit IgG (from an animal immunized with only the 48K and 55K components excised from SDS-PAGE gels) also eliminated collapsing activity (Figure 7a). In control experiments, immobilized preimmune IgG, which cross-reacts with somite proteins of 31K and 79K on Western blots, failed to eliminate this activity. SDS-PAGE analysis of glycoproteins that had been passed over preimmune IgG beads, bound to immune IgG beads, and then eluted from these in a pH 2.8 buffer revealed only two faint bands, of M_r 48K and 55K. These results indicate that among the glycoproteins obtained from somite tissue using immobilized PNA, the components of apparent M_r 48K and 55K are the major candidates bearing collapse activity.

Immunohistochemistry of Embryo Sections Using Affinity-Purified Antibody

Affinity-purified antibodies to the 48K and 55K components (see Figure 7b) stained only the posterior-half sclerotomes of stage 17–18 embryos (Figure 7c). This binding distribution exactly matched that seen with PNA (Figure 1a). In control experiments, using eluates from preimmune serum subjected to the same affinity purification procedure, no staining was seen.

Extraction with Immobilized Hyaluronate

To assess whether the components described here are related to hyaluronectin, a CNS-derived molecular complex with a high affinity for hyaluronate (see Discussion), detergent (2% CHAPS) extracts of stage 17–19 chick embryo trunks were passed over immobilized hyaluronate. This treatment failed to remove any collapse activity, and elution of the matrix with low pH also failed to recover any detectable protein. As a positive control, the immobilized hyaluronate was used to isolate hyaluronectin from a CHAPS-solubilized sample of adult human cortex. SDS-PAGE analysis of eluate from the hyaluronate beads showed bands of M_r 54K, 61K, and 64K.

Discussion

The lectin-based histochemical studies of chick somites described here confirm and extend the results of Stern et al. (1986). Binding of PNA to posterior-half sclerotome cells, as assessed by its staining pattern, appears immediately before the earliest outgrowth of both motor axons from the neural tube and sensory axons from the dorsal root ganglia and persists during outgrowth of later axons. Together with the fact that at least some of the PNA binding glycoconjugates are located on the surfaces of living P-cells, this observa-

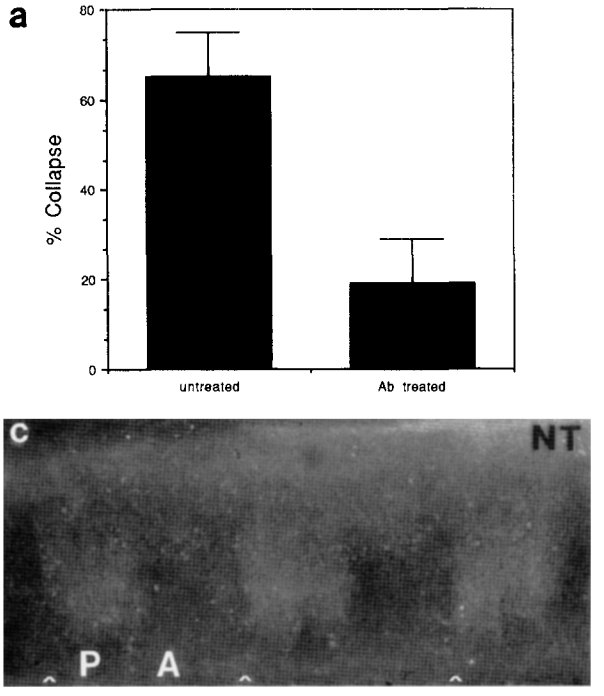

Figure 7. Antibodies to the 48K and 55K Components from PNA Affinity Chromatography Remove Collapse Activity

(a) Extracts in 2% (w/v) CHAPS in PBS of stage 17–19 chick trunks, when incubated at 4°C with preimmune IgG immobilized on Sepharose 4B, retain considerable quantities of collapse activity (69% ± 13%; untreated). However, immobilized IgG fraction containing antibodies to the 48K and 55K components isolated by immobilized PNA reduces collapse activity to control levels (29% ± 18%; Ab treated). Analysis of duplicate experiments using a paired t-test shows significant differences between untreated and Ab-treated samples at $P = 4.4 \times 10^{-2}$.

(b) A Western blot of stage 17–19 trunk proteins separated by SDS-PAGE and incubated with affinity-purified rabbit antibodies to the 48K and 55K components. Binding components are detected by autoradiography of blots subsequently treated with ^{125}I-labeled protein A. Consistently, only bands with apparent M_r 48K and 55K are seen. No bands are seen when preimmune serum, subjected to the same procedures, is used.

(c) A frozen sagittal section of a stage 19 chick embryo trunk stained with affinity-purified rabbit antibody and FITC-conjugated goat anti-rabbit IgG. Staining in the sclerotome is confined to the posterior half (P), with some staining also present in the neural tube (NT). Control sections treated with eluate from "affinity-purified" preimmune serum show no staining (data not shown). (A) Anterior-half sclerotome; arrowheads denote the segment boundaries. Magnification 100×.

tion suggests that these molecules are worth considering as agents that exclude axons from the posterior-half sclerotome. There is no evidence for a differential distribution of PNA binding glycoconjugates between the two halves of the youngest somites, both at the epithelial stage and during the early phases of somite disaggregation, when the neural crest cells begin their migration through the anterior-half sclerotome. In turn, this raises the possibility that the specific migration of neural crest cells into anterior-half sclerotome may be controlled by molecules other than those detected by the lectins used here.

Material binding to immobilized PNA has been characterized by SDS-PAGE. From material solubilized in CHAPS, up to eight protein bands are resolved under reducing conditions, two of which correspond to differences seen between the somite halves when their proteins are resolved on parallel tracks of SDS-PAGE gels. The fact that these two bands are so easily visible in one-dimensional separations indicates that these components are present in high abundance.

In two different in vitro assays, the components isolated from somites by the use of immobilized PNA are shown to inhibit neurite extension and the maintenance of a spread growth cone morphology. One assay is based on measurement of the extent of axon outgrowth on a substrate that has been coated with material eluted from immobilized lectin. The eluate reduces, but does not eliminate, axon extension from DRG. The remaining outgrowth may represent residual resistance to the presence of the inhibitory material; for example, growth cones may destroy it by the release of proteases (Krystosek and Seeds, 1981, 1984, 1986; Pittman, 1985).

The second assay examines the ability of the molecules in the eluate to cause growth cone collapse. Retraction of leading edge structures has been described most carefully by Kapfhammer and Raper (1987a, 1987b), who observed that contact of a PNS growth cone filopodium with a CNS axon, or vice versa, causes collapse of the entire growth cone. If, in vivo, the surfaces of posterior-half sclerotome cells are capable of eliciting an equivalent response, they would prevent advancing axons from entering the posterior-half sclerotome. Kapfhammer and Raper (1987a, 1987b) also observed that following growth cone collapse, neurites could sprout collaterally a short distance back along the axon shaft; a similar phenomenon in vivo could allow axons to find their way to the anterior-half sclerotome.

Subfractionation of the somite glycoproteins, using immobilized antibody, reveals that removal of the components of apparent M_r 48K and 55K, located exclusively in posterior-half sclerotome, eliminates collapse-inducing activity. Additional studies will be needed to determine whether one or both of these components are required to induce growth cone collapse and to establish their relationship to one an-

other in the native state. It is important here to compare these components with other known molecules that might also mediate the exclusion of nerve cells from the posterior-half sclerotome. To date, the only such candidate is cytotactin binding proteoglycan (CTB), a PNA binding glycoconjugate located in posterior-half sclerotome and suggested to be a controlling factor guiding neural crest cells into the anterior-half somite (Tan et al., 1987). CTB differs, however, from the active fraction described here in two important respects. First, even after chondroitinase treatment, when examined by SDS-PAGE under reducing conditions, CTB has an apparent M_r of 280K (Hoffman and Edelman, 1987; Hoffman et al., 1988). Second, using antibodies, CTB is found to be present initially in both sclerotome halves, only later localizing to the posterior-half sclerotome (Tan et al., 1987). Finally, Hoffman and Edelman (1987) have reported that hyaluronate is also present in their CTB preparations and suggest that it may have a binding function. Therefore, our observation that collapsing activity is unaffected by passage of somite extracts over immobilized hyaluronate also indicates that we are dealing with unrelated material.

In terms of molecular weight and tissue derivation, another molecule to consider is hyaluronectin, a glycoprotein isolated from adult human brain and also expressed in axial mesoderm during sclerotome/vertebra differentiation (Delpech and Halavent, 1981; Delpech and Delpech, 1984; Bignami and Dahl, 1986). Hyaluronectin binds specifically to hyaluronate in vitro, and its forms exist with molecular weights ranging from 45K to 110K. As described above, however, collapsing activity is not removed by adsorption of somite extracts with immobilized hyaluronate, suggesting further that the components described here are not related to hyaluronectin.

Other experiments examining the role of P-cells during spinal nerve segmentation have been reported. In those of Stern et al. (1986), axons from explants of chick stage 17 neural tube grew well on A-cells, but aggregated into bundles when growing on P-cells; growth cones were able, nevertheless, to grow on P-cells in these two-dimensional cultures, and it has been suggested that they might use regions of the P-cell surface seen to be devoid of PNA binding material. Tosney (1987, Soc. Neurosci., abstract) confronted chick motor axons extending in culture with P-cells and found that although neurites in general avoided these cells, they showed no evidence of contact paralysis. This observation might suggest that, in vivo, any growth cone response equivalent to the full collapse seen in vitro would involve only a part of the growth cone, for example, a single filopodium.

Directional inhibition of axon growth is becoming recognized as a mechanism of fundamental importance during neural development and regeneration (Verna, 1985; Kapfhammer et al., 1986; Stern et al., 1986; Kapfhammer and Raper, 1987a, 1987b; Walter et al., 1987; Caroni and Schwab, 1988a, 1988b; Patterson, 1988; Cox et al., 1990; Raper and Kapfhammer, 1990). In the present study, a PNA binding glycoprotein fraction derived from P-cells has been shown to result in growth cone collapse when applied in vitro. We suggest that one or both of these glycoproteins are responsible for the inhibition of growth of motor and sensory axons in the posterior half of the chick somite.

Experimental Procedures

Reagents and Solutions

Dulbecco's type A PBS (pH 7.3) was prepared from commercial tablets (Oxoid). Other solutions were as follows: TBS, 0.15 M NaCl, 0.05 M Tris (pH 7.4); TBB, 1% Triton X-100 in 50 mM sodium borate (pH 8.6); SDS-PAGE denaturing buffer, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.002% bromophenol blue, 10% (w/v) sucrose in water. DRG culture medium consisted of 5 μg/liter 7S NGF (Sigma) in 90% (v/v) F12 medium, 5% (v/v) fetal calf serum, 5% (v/v) chick serum (Flow), supplemented with 100 U/ml penicillin, 0.8 mg/ml streptomycin, and 0.25 μg/ml amphotericin (Sigma).

L-[^{35}S]methionine (1461 Ci/mmol) and D-[6-^{3}H]galactose (31.5 Ci/mmol) were obtained from Amersham International, Bucks, U. K. Fetuin (Spiro method) was purchased from GIBCO, and asialofetuin was produced as described previously (Bendiak and Cook, 1983).

Lectin Histochemistry

Fertilized hens' eggs (Comet Hubbard strain) were incubated at 37°C; embryos were staged according to Hamburger and Hamilton (1951). Embryos between stages 16 and 26 were ethanol-fixed, embedded in sucrose-gelatin, and sectioned with a cryostat (Stern et al., 1986). Sections were incubated in 1% (w/v) BSA (Cohn fraction V; Sigma) in PBS for 1 hr at room temperature and in a solution of FITC-PNA (3 mol of fluorescein per mol of lectin; Sigma) or FITC-Jacalin (2.6 mol of fluorescein per mol of lectin; Vector), both at 20 μg/ml, in PBS at 37°C for 4 hr. Sections were washed in 3 changes of 50 ml of PBS and mounted in Citifluor (City University, London). Controls were incubated in FITC-lectin in the presence of 0.2 M lactose. Sections were examined under epifluorescence with a Zeiss fluorescence microscope.

Lectin Staining of Isolated Cells

Stage 17-19 embryos were dissected to yield about 40 isolated half-somites. The half-somites were dissociated in ice-cold PBS containing 10 mM EDTA over the course of 1 hr, to yield separate suspensions of A- and P-cells (each half-somite, on dissociation, produces approximately 100 cells), and washed twice in 1 ml PBS at 8500 × g for 5 min at 0°C. Suspensions were placed in PBS containing 20 μg/ml FITC-PNA, incubated at 0°C for 30 min with occasional agitation, washed 3 times in 1 ml of PBS at 0°C, and viewed directly by epifluorescence microscopy.

Metabolic Labeling of Galactose-Containing Glycoconjugates from Somite Cells

Somite strips from up to 55 embryos (stages 17-19) were removed, and in each case the 5 somites at the anterior and posterior extremities were discarded. Strips were transferred to culture medium (DMEM; Flow Labs) supplemented with 2 μCi of D-[6-^{3}H]galactose, and incubated in 6% CO_2 at 37°C for up to 6 hr. For measurements of total ^{3}H incorporation, radiolabeled somites were washed 5 times in 1 ml of ice-cold PBS, resuspended in 0.5 ml of 2% (w/v) BSA in PBS, and homogenized at 0°C in a 1 ml Griffiths tube (BDH Chemicals). The homogenate was added to 10 ml of a 1% (w/v) solution of phosphotungstic acid in 0.5 M HCl and left on ice for 30 min. The precipitate was recovered by centrifugation at 480 × g for 10 min. The pellet was washed 4 times in 10 ml of PTA-HCl solution and solubilized in 1 ml of 1 M NaOH at 80°C. Aliquots (0.5 ml) of the solution were

mixed with 4.5 ml of Ecoscint (National Diagnostics) for scintillation counting.

Detergent Solubilization of Radiolabeled Somite Glycoconjugates

Somite strips were radiolabeled as above, washed in PBS, and divided into two equal parts, one of which was assayed for total incorporation of ^3H by precipitation in 1% (w/v) PTA-HCl. The other portion was solubilized in TBB, containing a mixture of 0.5 mg/liter leupeptin, 0.7 mg/liter pepstatin A, 1 mM EDTA, and 0.2 mM phenylmethylsulphonyl fluoride, on ice for 60 min using a Griffiths tube. This material was then centrifuged at 100,000 × g for 60 min at 4°C in a Sorvall OTD75B ultracentrifuge using a TST60.4 rotor, and the supernatant fluid was exhaustively dialyzed against TBB. A 0.5 ml aliquot of the dialyzed fluid was added to 4.5 ml of Ecoscint for scintillation counting.

Isolation of PNA Binding Glycoproteins by Affinity Chromatography

TBB-solubilized material from somites of 50 embryos, prepared as described above, was made 1 mM with respect to $CaCl_2$ and $MnCl_2$ before being passed through a 1 ml column of PNA immobilized on agarose beads (5 mg of PNA, capable of binding 5.5 mg of asialofetuin, per ml of settled gel; Vector). The column was washed in 10 ml of TBB containing 1 mM $CaCl_2$ and $MnCl_2$; bound glycoproteins were eluted in 10 ml of 0.2 M lactose in TBB. Column fractions of 0.7 ml were collected; 0.1 ml of each fraction was added to 4.5 ml of Ecoscint for scintillation counting.

SDS-PAGE of Affinity-Isolated PNA Binding Glycoproteins

PNA affinity chromatography was performed as above. The lactose-elutable material from five separate preparations was combined, dialyzed exhaustively against TBB diluted 10-fold with water, lyophilized, and redissolved by heating for 10 min at 100°C in SDS-PAGE denaturing buffer for analysis on slab gels (see below). In addition, some separations were performed in rod gels that were cut into 1 mm thick discs; these were then heated at 45°C for 48 hr in 0.5 ml of NCS tissue homogenizer (Amersham International); 0.5 ml of each solubilized disc was added to 4.5 ml of Ecoscint to confirm labeling of the 48K and 55K bands.

Comparison of Isolated Half-Sclerotomes by SDS-PAGE

Anterior- and posterior-half sclerotomes were dissected from 20 stage 17–18 embryos into ice-cold PBS, homogenized in SDS-PAGE denaturing buffer, and analyzed in 7.5% acrylamide slab gels (14 × 17 × 1 mm). The following proteins were used as molecular weight markers; carbonic anhydrase, ovalbumin, BSA, phosphorylase b, β-galactosidase, and myosin (Sigma). Proteins were visualized by silver staining (Morrissey, 1981).

Substrate Bioassay for Inhibition of Neurite Growth

Isolated DRG (from stage 33–35 embryos) were divided into two approximately equal parts and transferred onto 1 cm squares of Hybond N (Amersham International) previously treated for 30 min at room temperature with either a solution of 0.1% BSA in TBB or affinity-isolated PNA binding glycoprotein(s) and washed extensively in Hanks BSS. Following culture for 48 hr in 5 ml of DRG culture medium in 6% CO_2 at 37°C, the samples were fixed for 1 hr in 2% (w/v) formaldehyde in 15% (w/v) sucrose dissolved in PBS. After washing in PBS, cultures were stained with 0.2% toluidine blue, 40% (v/v) ethanol in water for 3 hr and destained with 70% (v/v) ethanol until individual axons could be distinguished. The extent of outgrowth of axons from the ganglia was measured using an eyepiece graticule on a Wild M50 dissecting microscope.

Preparation of Liposomes

Samples of the tissue to be studied, isolated by microdissection from about 60 embryos, were homogenized in 1 ml of solubilization buffer (2% [w/v] CHAPS [Sigma] in PBS) at 0°C in a 1 ml Griffiths tube. The homogenate was centrifuged at 100,000 × g for 60 min at 4°C to yield a supernatant fluid containing solubilized protein. Aliquots of 200 μl of the supernatant fluid were mixed with 200 μg of phosphatidyl choline (Sigma) and 20 μg of phosphatidyl serine (Sigma) dissolved in 20 μl of 4% (w/v) CHAPS in PBS, and dialyzed exhaustively against excess PBS at 4°C to form liposomes. Control liposomes were produced using 200 μl of solubilization buffer in place of the tissue extract. In addition, liposomes were prepared identically by incorporating 200 μl samples of affinity-purified (either PNA-or IgG-agarose; see below) material.

Samples of liposome suspensions were centrifuged at 100,000 × g for 2 hr at 4°C, and the pellet was subjected to freeze-fracture. Replicas were viewed by transmission electron microscopy on a Phillips EM300 at 60 kV, and calibration of magnification was performed using a fine graticule of known dimensions. Only replicas in which the "shadow" bisected the crater were used for measurements.

The efficiency of incorporation of PNA affinity-purified material into liposomes was assessed by adding tracer quantities of [^{35}S]methionine labeled PNA binding glycoproteins to unlabeled material, both having been isolated previously as described below. Metabolic labeling of somite polypeptides prior to affinity chromatography on PNA-agarose was carried out by the method of Lovell-Badge et al. (1985), using 16 somite strips (each consisting of 12 somites, the posterior somite of each strip lying 4 somites anterior to the segmental plate) dissected from stage 17–18 embryos.

Collapse Assay

Acid-washed, sterile, glass coverslips (13 mm diameter) were coated in pairs by sandwiching 40 μl of 30 μg/ml laminin (L4269; Sigma) in Hanks BSS between two coverslips, and placing them in 6% CO_2 for 1 hr at 37°C. After being washed in Hanks BSS, the coverslips were placed in 1 ml of DRG culture medium in wells of a 24-well plate (Flow). DRG were prepared for culture as described above, placed on the laminin-coated substrates, and grown for up to 20 hr.

Cultures were inspected with an inverted microscope, and those (about 75%) showing substantial growth, but few migrating nonneuronal cells, were selected for further experimentation. Each culture received 100 μl of liposome suspension, was warmed to 37°C, and was then incubated an additional for 1 hr. At this time, 2 ml of fixative (4% [w/v] formaldehyde, 15% [w/v] sucrose in PBS) was added to each culture, the top 2 ml of liquid (containing most of the original medium) was removed from each well, and the cultures were left for at least 6 hr at room temperature before being viewed under phase-contrast. Cultures were blind-coded before viewing. Only those axon termini that made no contact with other axons or cells were examined. Each terminus was scored as either "spread" (having the appearance of a typical growth cone, with small or large lamellipodia and/or filopodia) or "collapsed" (having none of the above).

Isolation of Collapse-Inducing Molecules Using Immobilized PNA

Stage 17–19 embryo trunks were solubilized in 2% (w/v) CHAPS in PBS as above, $CaCl_2$ and $MnCl_2$ were added to a final concentration of 1 mM, and 500 μl of the solution was mixed with 100 μl of either plain Sepharose 4B beads or Sepharose 4B beads coated with PNA (5 mg of lectin per ml of settled gel; Vector). The suspension of beads was incubated at 4°C overnight on a rotating mixer, and the unbound fraction was recovered by centrifugation at 8500 × g for 30 s. The beads were washed 10 times in 1.2 ml of 2% (w/v) CHAPS, 1 mM $CaCl_2$, 1 mM $MnCl_2$ in PBS and incubated in 500 μl of 0.4 M lactose, 2% (w/v) CHAPS in PBS for 5 hr on a rotating mixer at 4°C. The eluted material was recovered by centrifugation and dialyzed extensively against 2% CHAPS in PBS. Aliquots of samples were analyzed by SDS-PAGE, and liposomes were made from each sample by the method described above.

Immunization Protocol

The affinity-purified PNA binding fraction from 60 embryos was separated by SDS-PAGE. The gel regions containing the 48K and

55K bands were removed and emulsified in an equal volume of Freund's complete adjuvant (Sigma) and injected subcutaneously into a New Zealand White rabbit. Subsequent injections were prepared in Freund's incomplete adjuvant. Rabbits were bled routinely via the ear vein.

Isolation and Immobilization of IgG

IgG was isolated by sodium sulphate precipitation and ion exchange chromatography as described by Johnstone and Thorpe (1982), and purified material was shown by SDS-PAGE to contain only bands of M_r 22K and 49K, corresponding to IgG light and heavy chains, respectively. A yield of 850 μg of IgG was obtained from 5 ml of serum and was coupled to 0.3 g of CNBr-activated Sepharose 4B (Sigma); any remaining binding sites were blocked with ethanolamine. In this work, >800 μg of IgG was immobilized per ml of gel.

Affinity Purification of Antibodies

Affinity-purified antibodies against the 48K and 55K bands were isolated from the serum using the procedures adopted by Koch et al. (1986) and Zalik et al. (1987). Briefly, the region of a Hybond C (Amersham) blot of somite proteins, containing the 48K and 55K bands, was cut out and incubated in 10% (w/v) BSA, 1% (w/v) dried skimmed milk in PBS for 1 hr at 4°C, washed in 50 ml of PBS, and incubated in 5 ml of immune serum overnight at 4°C on a rocker. The blot was then washed for 2 hr in 6 changes of 20 ml of PBS and immersed in 3 ml of 0.1 M glycine-HCl (pH 2.8) for 10 min at 4°C. The antibody solution was neutralized with solid disodium hydrogen orthophosphate and dialyzed extensively against many changes of 4°C PBS.

Western Blot Analysis Using Antibodies

Proteins (from the somites of 10 embryos per track of gel) were separated using SDS-PAGE on 7.5% gels and blotted onto Amersham Hybond C-extra using an LKB Novablot semi-dry apparatus. The blots were immersed for 2 hr at 4°C in 10 ml of 10% (w/v) BSA and 1% (w/v) dried skimmed milk in PBS to saturate any nonspecific protein binding sites and washed twice in 50 ml of PBS. Solutions of antiserum (1/50), or affinity-purified antibody (1/5) were made in 5 ml of 10% (w/v) BSA, 1% (w/v) dried skimmed milk in PBS and placed on strips of the blot on a rocker at 4°C for 12 hr. The strips were washed for 1 hr in 3 changes of 50 ml of PBS, incubated with ^{125}I-labeled protein A, washed in 3 changes of 50 ml of PBS, and autoradiographed at −70°C using Fuji RX film and a tungstate intensifying screen.

Immunohistochemistry Using Affinity-Purified Antibody

Frozen sections of embryos, which had been fixed in 2% (w/v) formaldehyde in PBS, were incubated in 1% (w/v) BSA, 0.1% dried skimmed milk at 4°C for 2 hr, washed in PBS, and incubated in a 1:3 dilution of affinity-purified rabbit antibody in 1% (w/v) BSA, 0.1% dried skimmed milk for 8 hr. Following 2 washes in 50 ml of PBS for 1 hr at 4°C, sections were incubated in 20 μg/ml FITC-goat anti-rabbit IgG (affinity-isolated; Sigma) in 1% BSA, 0.1% dried skimmed milk in PBS for an additional 8 hr, washed in 50 ml of PBS for 2 hr at 4°C, and mounted in Citifluor as above.

Preparation of Immobilized Hyaluronate

Hyaluronic acid (from human umbilical cord; Sigma grade 1) was coupled to AH-Sepharose 4B by the method of Tengblad (1979), with the exception that the coupling reaction was allowed to proceed over 6 hr. Uronic acid analysis (Bitter and Muir, 1962) of both original hyaluronate solution and material recovered after coupling to AH-Sepharose 4B gave a concentration of immobilized hyaluronate of 4.6 mg per ml wet gel.

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