Biochemical Characterization of Netrin-synergizing Activity

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The netrin-1 protein elicits spinal commissural axon outgrowth and turning in vitro and has been shown to be required for commissural axon guidance in vivo in the developing spinal cord. Biochemical observations made during the purification of netrin-1 suggest that this ligand and its receptor, DCC, may not function alone in directing commissural axon guidance. Recombinant netrin-1 protein is ∼10 times more active in eliciting axon outgrowth from embryonic day (E) 13 rat dorsal spinal cord explants than from E11 rat dorsal spinal cord explants (Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M., and Tessier-Lavigne, M. (1994) Cell 78, 409–424) even though the starting material for the netrin purification, a high salt extract of E10 chicken brain membranes, is equally active on E13 and E11 explants. We previously reported an activity termed netrin-synergizing activity (NSA) that can potentiate the outgrowth-promoting activity of netrin-1 on E11 explants (Serafini et al.). Here we report a biochemical characterization of NSA in netrin-depleted high salt extracts of E10 chicken brain membranes. We provide evidence that NSA is composed of a denaturation-resistant basic protein(s) in the 25–35-kDa size range. We also provide evidence that the activity may be heterogeneous, splitting into three species that may be distinct or related. The results reported here should facilitate purification of this activity from a more abundant source or identification of the activity based on similarity to known proteins that share its distinctive biochemical properties.

The faithful guidance of axons to their targets during development of the embryonic nervous system is believed to occur through the action of both positive and negative guidance factors (1, 2). These factors may act either locally or at a distance within the terrain of the developing embryo. A large body of experimental evidence has implicated the phylogenetically conserved netrin gene family in directing the long-range attraction of circumferentially migrating neurons in Caenorhabditis elegans, Drosophila, and vertebrates (reviewed in Ref. 1). In vertebrates, the netrin-1 mRNA is expressed in the floor plate, the ventral midline intermediate target of migrating commissural neurons, before and during the migration of these neurons toward this structure (3). The netrin-1 receptor on migrating commissural neurons is the netrin-binding DCC (deleted in colorectal cancer) protein, which is expressed on commissural axons as they migrate toward the floor plate (4). Mutant mice deficient for either the netrin-1 gene product or DCC exhibit defects in commissural axon migration from their birthplace in the dorsal spinal cord to the floor plate, demonstrating that their proper functioning is necessary for commissural axon guidance (5, 6).

The vertebrate netrin-1 protein was originally purified from a high salt extract of embryonic day (E) 10 chicken brain membranes on the basis of its ability to mimic the axon outgrowth-promoting effect of floor plate extracts from dorsal spinal cord explants cultured in vitro within three-dimensional collagen matrices (7). During this purification, an activity was discovered that collaborates with netrin-1 to promote outgrowth. Although recombinant netrin-1 protein is ∼10 times more active in eliciting axon outgrowth from E13 rat dorsal spinal cord explants than from E11 rat dorsal spinal cord explants, a high salt extract of E10 chicken brain membranes is equally active on E13 and E11 explants. We show that the difference is due to the presence in brain extracts of a distinct activity, netrin-synergizing activity (NSA), that is capable of potentiating the axon outgrowth-promoting effects of netrin-1 from E11 explants (7). Because of its dramatic potentiation of netrin-mediated axon outgrowth in vitro, it seems likely that this activity may play an important role in commissural axon guidance in vivo. As a first step toward identifying NSA and determining its role in commissural axon guidance in vivo, we report here the results of a biochemical characterization of NSA from a netrin-depleted high salt extract of E10 chicken brain membranes.

EXPERIMENTAL PROCEDURES

Synergy Assay and Culture of E11 Dorsal Spinal Cord Explants—E11 rat dorsal spinal cord explants (E0 = day of vaginal plug) were dissected as described (8) with the protease digestion protocol described previously (7). Explants were cultured for 40 h at 37 °C and 5% CO2 in 50% nutrient mixture F-12 and 38.25% Opti-MEM containing 40 μg glucose, 5% heat-inactivated horse serum, 1% penicillin/streptomycin, and 1% Glutamax-1 additive (Life Technologies, Inc.). For most assays, a concentrated high salt extract of stably transfected netrin-expressing cells (9) was diluted 133-fold into the above culture medium to give a final netrin-1 concentration of ∼50 ng/ml in the culture medium. Control explants without netrin-1 were cultured with an equivalent dilution of 1.0 M NaCl and 20 mM Na2HPO4 (pH 7.0).

Immunostaining—Explants were fixed after culture for 1 h with 4% paraformaldehyde at room temperature, washed with phosphate-buffered saline, and then blocked with phosphate-buffered saline containing 1% heat-inactivated normal goat serum and 0.1% Triton X-100. Analysis of TAG-1 expression was performed with a 1:100 dilution of monoclonal antibody AF5 (Oncogene Science, Inc.), followed by horseradish peroxidase/hematoxylin staining. Detection of DCC expression was performed with a 1:100 dilution of monoclonal antibody AF5 (Oncogene Science, Inc.), followed by horseradish peroxidase/hematoxylin staining (Sigma). The difference in the detection reagents accounts for the difference in the color of labeled axons in Fig. 1 (E and F).

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1 The abbreviations used are: E, embryonic day; NSA, netrin-synergizing activity; PAG, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TEMED, N,N,N’,N’-tetramethylethlenediamine.
Quantification of Axon Outgrowth—Axon fascicles emerging from each explant were measured and added together to determine the total bundle length of these fascicles per explant. Four to six explants from each condition in three independent experiments were counted. Thin single neurones, which were rare, were not counted.

The Supernatant of the Synergy Assay—Factors were tested in one or more of the following three ways: 1) as pure protein serially diluted in assay medium (generally 2-fold dilutions from 1 μg/ml to 0.5 ng/ml), 2) as conditioned medium harvested from cells 48 h after transfection with an expression plasmid for the factor of interest, and 3) from clumps of cells transfected with an expression plasmid for the factor of interest and position of one or more of the following: 1) a 150 mM NaCl, 20 mM Na2HPO4 (pH 7.0) was heated for 15 min at 95 °C, followed by 10 min on ice. A control sample was left at room temperature for 15 min, the supernatant was then dialyzed against nutrient mixture F-12 for 1 h at 4 °C to remove precipitated proteins and stored at −80 °C before neutralization to pH 7.0 with 5M NaOH. This material was then dialyzed at 4 °C for 2 h at room temperature against 4 liters of acid/urea sample loading buffer containing 6M urea and 250 mM sodium acetate (pH 5.8). Finally, a 1:2000 dilution of TEMED was used to start the polymerization. A five-well comb was inserted above the gel; there was no stacking gel. Prior to loading the sample, the gel was prerun at room temperature for 1 h at 150 V of constant voltage to remove charged species from the gel. The supernatant from this spin was then dialyzed for 2 h at room temperature against 4 liters of acid/urea sample loading buffer containing 6 μM urea and 250 mM sodium acetate (pH 5.8). Finally, a 1:2000 dilution of TEMED was used to start the polymerization. A five-well comb was inserted above the gel; there was no stacking gel. Prior to loading the sample, the gel was prerun at room temperature for 1 h at 150 V of constant voltage to remove charged species from the gel.
shown). Each strip was minced with a clean razor and transferred into 400 μl of 6 μm urea in one well of a four-well tissue culture dish precoated with 10% serum for several hours to block the protein-binding capability of the dish. The dishes were shaken overnight at room temperature to elute protein from the minced slices. The following morning, the elution buffer was recovered from each well, and a portion of each fraction was analyzed by SDS-PAGE. The rest of each fraction was dialyzed for several hours against 5.4 liters of nutrient mixture F-12 for subsequent analysis in the synergy assay.

Reverse-phase Chromatography—7 ml of NSA-containing heparin eluate was first prepared for chromatography by heating as described above. The supernatant was then dialyzed for 2 h against a 1000-fold volume excess of unbuffered water. The dialysate was spun in a minidrydown in a SpeedVac, resuspended in 150 mM NaCl and 100 mM Tris-Cl (pH 8.0) to neutralize residual acid, dialyzed against nutrient mixture F-12 and serially diluted into dialysis medium for assay. The remaining 25% of each fraction was dried down without carrier protein and used for the Bio-Rad/Bradford protein assay and analysis of the fractions by SDS-PAGE.

**RESULTS**

**Initial Characterization of NSA**—We previously demonstrated that netrin-depleted high salt extracts of E10 chick brain membranes contain an activity (NSA) that can potentiate netrin-mediated axon outgrowth from explants of E11 rat dorsal spinal cord (7). In those experiments, the addition of brain extracts caused necrosis within the explants, resulting in variability of responses (data not shown). We found, however, that reproducible non-necrotic growth of E11 dorsal spinal cord in the presence of complex protein fractions from chicken brain was obtained if the cultures were performed in a mixture of nutrient mixture F-12/Opti-MEM supplemented with a stable glutamine derivative, Glutamax-1 (Fig. 1, A–D). E11 explants grown alone, in the presence of ~50 ng/ml recombinant netrin-1 protein, or in the presence of a heparin flow-through fraction containing NSA exhibited little or no commissural axon outgrowth into the surrounding collagen (Fig. 1, A–C). Explants grown in the presence of both ~50 ng/ml netrin-1 protein and NSA exhibited robust axon outgrowth primarily but not exclusively from the ventral cut edge of the dorsal spinal cord explants (Fig. 1, D–F).

The rat dorsal spinal cord explants employed in the synergy assay contain both commissural neurons and association neurons. Commissural neurons are distinguishable by virtue of their expression of the axonal glycoprotein TAG-1 (14) and the netrin receptor, DCC (4). Most or all of the responsive axons in the synergy assay are commissural neurons since they were labeled by antibodies directed against the axonal glycoprotein TAG-1 (Fig. 1E) and the netrin receptor, DCC (Fig. 1F).

The commissural axons present in the E11 explants responded to the NSA-containing heparin flow-through fraction in a dose-dependent fashion in the presence of a constant amount (~50 ng/ml) of recombinant netrin-1 protein. NSA exhibited a maximal outgrowth response at 0.26 mg/ml heparin flow-through fraction. The dose-response curve for NSA was also very steep, showing minimal commissural axon outgrowth at one-quarter the peak concentration as well as a diminished response beyond the peak concentration (Fig. 1G).

The assay described above provides a useful tool for a possible biochemical purification of NSA. In this synergy assay, E11 explants are grown within three-dimensional collagen matrices in the presence of ~50 ng/ml netrin-1 protein, a netrin-1 concentration that elicits minimal axonal outgrowth from these explants. The NSA-containing heparin flow-through starting material is then fractionated. Serial dilutions of the generated fractions are dialyzed against nutrient mixture F-12 and added to the explants with netrin-1, cultured for 40 h in three-dimensional collagen matrices, and then scored for the outgrowth of axonal fascicles. For the quantification of the biochemical fractionation experiments detailed below, we did not routinely perform the laborious axonal length measurements presented in Fig. 1G. Rather, we defined a unit of activity as the lowest dilution of any given fraction dissolved in 1 ml of assay medium...
that would give a maximal response (similar to that shown in Fig. 1, D–F) in the synergy assay.

**NSA Is Not Encoded by a Variety of Known Extracellular Factors and, Like Netrin-1, Is a Heparin-binding Protein—**

Before embarking on a potentially laborious biochemical purification of NSA, we tested a number of known factors to determine whether they might encode NSA-like activity. These factors were chosen on the basis of one or more of the following criteria: 1) known effects on the in vitro growth of other classes of neurons, 2) expression in the embryonic spinal cord, and 3) similarity to the biochemical properties of NSA (see below). Candidates tested (Table I) included neurotrophic factors, extracellular matrix components, growth factors, extracellular proteases, and protease inhibitors. None of the proteinaceous factors tested to date exhibited any NSA-like activity.

The addition of 2 M urea and 0.3% CHAPS detergent was required to keep NSA soluble and stable in low salt solutions.

### Table I

| Factors tested for netrin-synergizing activity |
|-----------------------------------------------|
| F-spondin<sup>a,b</sup>                        |
| Sema3A<sup>c</sup>                            |
| Sema3C                                         |
| Sema3F                                         |
| Stem cell factor (steel factor)<sup>a,c</sup>  |
| Hepatocyte growth factor<sup>a</sup>           |
| Decorin<sup>a</sup>                           |
| Tissue plasminogen activator<sup>a</sup>       |
| Nerve growth factor<sup>a</sup>                |
| Neurotrophin-3<sup>a</sup>                     |
| Neurotrophin-4/5<sup>a</sup>                   |
| Brain-derived neurotrophic factor<sup>a</sup>  |
| Ciliary neurotrophic factor<sup>a</sup>        |
| Neuregulin<sup>a</sup>                        |
| Basic fibroblast growth factor<sup>a,c</sup>   |
| Epidermal growth factor<sup>a</sup>           |
| TIMP-1<sup>a,c</sup>                          |
| TIMP-2<sup>a,c</sup>                          |
| TIMP-3<sup>a</sup>                            |
| Matrix metalloprotease-2<sup>a</sup>           |
| Dorsalin<sup>a</sup>                          |
| Bone morphogenetic protein-7<sup>a</sup>       |

<sup>a</sup> Tested as pure protein.

<sup>b</sup> Tested as conditioned medium from transfected cells.

<sup>c</sup> Tested from transfected cell aggregates.

<sup>d</sup> TIMP, tissue inhibitor of metalloproteases.

<sup>e</sup> Causes axonal outgrowth qualitatively distinct from that observed with NSA.

**Netrin-synergizing Activity**

In the presence of these additives and 150 mM NaCl, NSA did not bind to anion exchangers (Q-Sepharose) and did bind to cation exchangers (S- and CM-Sepharose) and to heparin-Sepharose, a cationic affinity resin. The majority of NSA in our starting material, when depleted of anionic proteins on Q-Sepharose, bound to heparin-Sepharose in the presence of 2 mM urea, 0.3% CHAPS, 150 mM NaCl, and 20 mM Tris-Cl (pH 8.0) and eluted from this resin in a broad peak from ∼500 to 800 mM NaCl (Fig. 2). With some batches of material (the run shown in Fig. 2 is an example of this), the activity peak was broader than this, with up to one-third of the activity eluting in lower salt fractions. Even when the activity eluted more broadly, we consistently pooled only fractions 7–10 for further biochemical analysis in order to avoid abundant contaminant proteins present in the lower salt fractions. Pooled active fractions were dialyzed against 1 mM NaCl and 20 mM Na₂HPO₄ (pH 7.0) to stabilize NSA and were stored at −80 °C until further biochemical analysis. We sometimes saw, as in Fig. 2, what looked to be two peaks of activity eluting from the heparin column. These results indicate that NSA, like netrin-1, is also a heparin-binding protein(s), although it elutes at lower salt than netrin-1 (500–800 mM NaCl for NSA compared with ∼1.2 mM NaCl for netrin-1). Note that NSA is present in the flow-through fraction from the heparin column in the netrin purification (7) because that column is loaded at 0.9 mM NaCl.

NSA Is a Protein(s) Extremely Resistant to Denaturation—

Pilot experiments with a number of chromatographic techniques (lectin affinity chromatography, metal affinity chromatography, high resolution ion-exchange, hydrophobic chromatography, dye chromatography, hydroxylapatite chromatography, and netrin affinity chromatography) demonstrated these techniques to be ineffective in resolving NSA in our partially purified heparin eluate fraction from the bulk of the remaining protein (<2.5-fold purification was obtained with any of the above techniques), indicating that purification of NSA from our limited source material might not be feasible (data not shown). Further characterization of NSA was facilitated, however, by the observation that NSA is particularly resistant to denaturing conditions. NSA present in the partially purified heparin eluate survived exposure to SDS, trifluoroacetic acid, heat (95 °C), and 6 mM urea (Table II). In addition, NSA present in the pooled heparin eluate fractions was stable.

**Fig. 2.** Ion-exchange/heparin affinity chromatography of the NSA-containing heparin flow-through fraction. The NSA-containing heparin flow-through fraction was dialyzed against equilibration buffer and then centrifuged to remove precipitated protein and nucleic acid. The material was loaded onto 5-ml Q and heparin columns linked in tandem (with the Q column in first) on a fast protein liquid chromatography system. After loading, the Q column was removed, and the bound protein was eluted from the heparin column with a 50-ml linear gradient from 150 mM to 1.0 M NaCl in the presence of 2 mM urea, 0.3% CHAPS, and 20 mM Tris-Cl (pH 8.0). 5-ml fractions were collected, and the protein concentration (blue line; mg/ml), conductivity (red line; millisiemens (mS/cm)), and netrin-synergizing activity (black bars; units/ml) were determined for each fraction. The data represent the results from one such run in which NSA eluted broadly from the heparin column (see “Results”). Fractions 7–10 from this and other runs were pooled for further biochemical analysis.
TABLE II
Stability of NSA to denaturing conditions

| Test condition         | Activity recovery (%) |
|------------------------|-----------------------|
| Water                  | 60                    |
| Water + 0.1% TFA (pH 1.6) | 60                  |
| Water + 0.5% SDS       | 45                    |
| Heat (95°C in 1 M NaCl) | 40–80*               |
| 6 M urea               | 75                    |
| Protease               | <12                   |

*TFA, trifluoroacetic acid.

*Stability of NSA to heat varies from preparation to preparation of the heparin eluate.

FIG. 3. Recovery of activity from nonreducing SDS-polyacrylamide gels demonstrates that NSA is most likely between 25 and 35 kDa and is encoded by a minor protein species. The NSA-containing heparin eluate was prepared for nonreducing SDS-PAGE as described under “Experimental Procedures.” Portions of each fraction from the nonreducing gel were used for serial dilution in the synergy assay to quantitate netrin-synergizing activity (bar graph) and for reducing SDS-PAGE and silver staining to visualize the protein complexity of each fraction (gel). The numbers below the gel indicate the fraction numbers. Molecular mass markers (in kilodaltons) are indicated to the left of the gel. Activity was centered on fractions 2 and 3, with some activity also in fraction 4. These fractions contain proteins in the 25–35-kDa size range. Examination of these proteins and, in particular, comparison of fractions 2 and 3 showed that none of these major proteins were distributed among the fractions in a manner that is consistent with their encoding the activity indicated by the graph. Furthermore, no activity resided with the lower molecular mass proteins in fraction 1 or in the higher molecular mass fractions 5 and 6.

FIG. 4. Native acid/urea electrophoresis of the NSA-containing heparin eluate. The NSA-containing heparin eluate was heated, centrifuged, dialyzed against water, re-centrifuged, concentrated, and dialyzed into native electrophoresis sample buffer as described under “Experimental Procedures.” After electrophoresis on the native acid/urea gel (see “Experimental Procedures”), the gel was cut into slices, which were numbered from the bottom of the gel up, and protein was eluted from each slice. A portion of each fraction was analyzed by reducing SDS-PAGE and silver staining to assess the protein complexity of each fraction (gel), and another portion was serially diluted in the synergy assay to assess the amount of netrin-synergizing activity in each fraction (bar graph). Activity was observed in fractions 4 and 5. Numbers below the gel indicate the fraction numbers; Hep refers to the starting heparin eluate fraction, and L refers to this fraction after heating and dialysis against water in preparation for electrophoresis. Molecular mass markers (in kilodaltons) are indicated to the left of the gel. The band present in all fractions between 45 and 66 kDa is a carrier protein added during elution of protein from each of the gel slices.

Recovery of NSA from Nonreducing SDS-Polyacrylamide Gels Indicates That NSA Is a Minor Protein Species That is Most Likely in the 25–35-kDa Size Range—We took advantage of the SDS stability of NSA to fractionate the proteins present in our partially purified heparin eluate by nonreducing SDS electrophoresis, reasoning that the dissociative properties of SDS would give us a more accurate size estimate than conventional gel filtration. The NSA-containing heparin eluate was prepared for electrophoresis as described under “Experimental Procedures.” The proteins were then separated on a 10% polyacrylamide gel. The gel was cut into strips (fractions were numbered from the bottom up) with a razor blade, and the proteins were eluted from the minced strips by passive diffusion into 6 M urea. After removal of SDS by a modification of the technique of Weber and Kuter (11), the fractions were split into portions that were either rerun on a reducing SDS-polyacrylamide gel that was subsequently silver-stained to assess protein complexity or assayed in the synergy assay to detect NSA. The results of this analysis on one such gel are shown in Fig. 3. All of the activity was found to reside in three fractions that contained proteins centered around 30 kDa. Lower molecular mass proteins present in fraction 1 were inactive, as were higher molecular mass fractions, only some of which are shown. Furthermore, comparison of the intensity of visible protein bands in the active and inactive fractions and particularly between fractions 2 and 3, which possess equivalent activity, demonstrated that NSA is not encoded by any of the major protein species in these fractions. Due to a very low recovery of NSA and gel-to-gel variability in the separation of NSA with respect to the abundant surrounding contaminant bands, it was not feasible, given our limited starting material, to employ this technique for purification of NSA. Nevertheless, our data strongly suggest that NSA resides in a minor protein(s) in the size range of 25–35 kDa (although we cannot exclude that some activity may also be...
associated with smaller protein species that were run off of this gel or that NSA, due to its basic nature (see below), might migrate at an aberrantly high molecular mass on nonreducing SDS-PAGE.

Fractionation of the NSA-containing Heparin Eluate by Native Acid/Urea Electrophoresis—Despite the low recovery of protein and activity on nonreducing SDS-PAGE, we hoped that another electrophoretic technique run under different conditions might prove more useful in the fractionation of NSA. In particular, we wished to investigate native electrophoresis in the presence of acid and urea, a technique that is ideal for separating basic proteins from one another (12, 13). We assumed that the activity was a basic protein(s) based on its behavior on ion-exchange/heparin affinity resins (Fig. 2). The results of one native electrophoresis experiment are illustrated in Fig. 3. The partially purified NSA-containing heparin eluate was prepared for electrophoresis as described under “Experimental Procedures.” After separation on the polyacrylamide gel, the gel was cut into strips (which were numbered from the bottom of the gel up and minced with a clean razor blade). Portions of each fraction were either rerun on a reducing SDS-polyacrylamide gel and then silver-stained to assess protein complexity or assayed in the synergy assay to detect NSA. The complexity of these fractions is less than that observed on nonreducing SDS-PAGE in Fig. 3 because the material loaded onto this gel was from a batch of the heparin eluate that could be (and was) heated with only a small loss of activity. Comparison of the protein complexity of each fraction revealed that the proteins on the acid/urea gel were not separated solely as a function of their size (as expected for a technique that fractionates by a complex function of charge and mass). Robust activity was recovered in fractions 4 and 5, which contain a set of proteins centered around 30 kDa. This result demonstrates conclusively that NSA is encoded by a basic protein(s) since upon application of the electric field in the presence of the highly dissociative 6 m urea, only basic proteins should migrate through the acid/urea gel. Unfortunately, this technique is also plagued by the low activity recovery problems we encountered with nonreducing SDS-PAGE; and therefore, its use in a purification scheme was precluded because the number of embryonic chicken brains required to include it as a purification step was prohibitive.

Multiple Peaks of NSA Eluate from Reverse-phase Columns—The stability of NSA to trifluoroacetic acid suggested that reverse-phase chromatography might prove useful for the fractionation of this activity. The NSA-containing heparin eluate was prepared for C4 reverse-phase chromatography (as described under “Experimental Procedures”). The column was developed with a gradient of increasing acetonitrile concentration. Samples from each fraction from the C4 column were analyzed in the synergy assay as well as by SDS-PAGE and silver staining to examine the protein complexity of the fractions (Fig. 5). Electrophoresis revealed separation of the remaining proteins that was far better than that observed with other non-electrophoretic chromatographic techniques (Fig. 5 and data not shown). Synergy assays revealed the presence of

**Table III**

| Fraction                        | Volume | Total protein | Total activity | Specific activity | Fold Recovery |
|--------------------------------|--------|---------------|----------------|-------------------|---------------|
| Heparin flow-through<sup>a,c</sup> | 72     | 79.2          | 288            | 260               | (1)           |
| Q flow-through/heparin eluate<sup>d</sup> | 7      | 7.9           | 192            | 40                | 6.5           |
| Heated heparin eluate<sup>d</sup> | 7      | 2.3           | 92             | 25                | 10.4          |
| C4 reverse-phase               |        |               |                |                   |               |
| Total pool<sup>ab</sup>         | 8      | 1.0           | 48             | 20                | 13.0          |
| Peak I<sup>c</sup>              | 3      | 0.4           | 21             | 18                | 14.4          |
| Peak II<sup>d</sup>             | 2      | 0.4           | 18             | 20                | 13.0          |
| Peak III<sup>d</sup>            | 2      | 0.2           | 6              | 30                | 8.7           |

<sup>a</sup> Owing to the semiquantitative nature of the quantification method employed (see “Experimental Procedures” and “Results”), all unit measures and values calculated using these measurements are subject to a 2-fold uncertainty.

<sup>b</sup> Although this fraction has already been significantly enriched from the purification source (embryonic chicken brains), it is the first fraction in which NSA could be measured independently of netrin and therefore was considered the starting material for the purification of NSA.

<sup>c</sup> For ease of comparison, the amounts of these fractions have been normalized to the amount of material used for the C4 reverse-phase chromatography run shown in Fig. 5 and analyzed here, ~750 E10 chicken brains. In practice, the Q/heparin step was run with ~2000 brains'-worth of heparin flow-through starting material, as in Fig. 2.

<sup>d</sup> As indicated in Fig. 2, the Q flow-through/heparin eluate consists of pooled fractions 7–10 from this run. The values specified here are for these pooled fractions only.

The C4 reverse-phase total pool is the summed protein and activity of fractions 21–26, 28, and 29 of the run shown in Fig. 5. Peak I consists of fractions 21–23; peak II consists of fractions 25 and 26; and peak III consists of fractions 28 and 29.
three distinct peaks of NSA (Fig. 5). The presence of these three peaks was confirmed by multiple runs with different batches of the NSA-containing heparin eluate (data not shown). Comparison of the protein profiles of the active fractions revealed that they contain largely nonoverlapping proteins with the exception of band(s) in the ~30-kDa range (Fig. 5 and see “Discussion”). Table III gives a quantitative assessment of the most useful chromatography steps we have identified to date, including the ion-exchange/heparin step shown in Fig. 2 and the reverse-phase chromatography shown in Fig. 5. The modest -fold purification afforded by these steps is insufficient for purification to homogeneity from our current source, but we hope that future fractionation of NSA(s) present in a more easily obtained starting material will allow molecular identification of these activities, which may be related or distinct molecular species.

**DISCUSSION**

Axon guidance cues can be classified into four categories: positive or negative cues that act either locally or at a distance to guide axons within the developing embryo (1, 2). The fact that NSA does not have any axon outgrowth-promoting activity on its own suggests that this activity may be acting in a mechanistically distinct manner from that of previously identified positive and negative axon guidance factors, through modulating the activity of a known axon guidance cue. It will be difficult to unravel the mechanism of action and in vivo function of this cue without its molecular identification. The results presented here should aid in that endeavor. Here we describe an improved assay for NSA and the use of this assay to define the biochemical properties of NSA. Fractionation of NSA reveals a number of important observations that should facilitate future molecular identification. First, despite its remarkable stability to a variety of denaturing conditions, this activity contains a necessary protein component for activity since it is abolished by protease treatment. This observation, coupled with the finding that NSA is a basic protein (indicated by the behavior of NSA on ion-exchange resins and on native acid/urea electrophoresis), indicates that NSA is not encoded by a glycosaminoglycan or proteoglycan moiety as was originally hypothesized (7) since brain-derived proteoglycans (but not NSA) bind to anion exchangers at 150 m mM NaCl (15).

It appears that the NSA present in our starting material, a netrin-depleted high salt extract of embryonic chick brain membranes, may be heterogeneous, as indicated by the existence of multiple activity peaks that elute from a C4 reverse-phase chromatography column. All of these peaks must be encoded by basic proteins (since they all elute from heparin at basic pH), and they are likely to all be in the same size range (25–35 kDa) since this is where all NSA-like activity is recovered in nonreducing SDS electrophoresis experiments. One could argue that some of these NSAs are stable to trifluoroacetic acid and not to SDS (a possibility our stability experiments do not address) and that we therefore really know the size of only one of the three NSAs. Although this is possible, we think it is unlikely since resistance to one denaturing condition generally implies a structure that imparts resistance to other denaturing conditions. Examples of this principle include the neurogulins (16) and the tissue inhibitors of metalloproteases (17), both of which survive exposure to a number of denaturing conditions. The most likely interpretation of our nonreducing electrophoresis and reverse-phase chromatography experiments is that each peak observed on reverse-phase chromatography is in the 25–35-kDa size range we defined for NSA by nonreducing SDS-PAGE.

Most of the difficulty in purifying this activity can be attributed to the facts that we have not been able to identify a high enrichment affinity chromatography step specific for this activity (since neither a panel of lectins nor netrin affinity resins bind NSA) and that the steps we have identified so far give only modest enrichment of NSA (Table III). Published biochemical purifications of other axon guidance molecules have all included affinity steps and may not have been possible without them (7, 18, 19). Through future fractionation experiments, we hope to determine whether the three activity peaks we observed with reverse-phase chromatography represent independent proteins, related but distinct proteins, or a single protein with several different modifications. Our suspicion, based on the results of nonreducing SDS-PAGE of NSA-containing fractions, is that NSA is encoded by minor protein species present in trace amounts in our fractions. Mass spectrometry and Edman degradation sequencing of the most abundant protein species that approximately cofractionate with our observed activity on reverse-phase chromatography (the ~30-kDa bands shown in Fig. 5) bear this conclusion out; to date, the proteins we have sequenced from these fractions are all highly basic histone and ribosomal proteins. Although these proteins share biochemical properties with NSA, their intracellular nature makes it unlikely that they actually encode NSA. Due to low recoveries of NSA on reverse-phase chromatography, the lack of an affinity chromatography step, and the fact that NSA in embryonic chicken brain appears to be present in trace amounts, purification of NSA from embryonic chicken brain does not seem currently feasible. Identification of an affinity chromatography step or purification from a more abundant activity source (perhaps adult chicken or bovine brain) may surmount these obstacles to the molecular identification of NSAs.

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* W. Henzel, C. Tuckr, M. Tessier-Lavigne, and M. J. Galko, unpublished observations.
