The Axonally Secreted Protein Axonin-1 Is a Potent Substratum for Neurite Growth

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Abstract. Axonin-1 is a neuronal glycoprotein occurring both as a membrane-bound and a secreted form. Membrane-bound axonin-1 is predominantly located in membranes of developing nerve fiber tracts and has recently been characterized as a cell adhesion molecule; the soluble form is secreted from axons and accumulates in the cerebrospinal fluid and the vitreous fluid of the eye. In the present study, we addressed the question as to whether secreted axonin-1 was released in a functionally competent form and we found that it strongly promotes neurite outgrowth when presented to neurons as an immobilized substratum. Neurite lengths elaborated by embryonic dorsal root ganglia neurons on axonin-1 were similar to those on the established neurite-promoting substrata L1 and laminin. Fab fragments of axonin-1 antibodies completely inhibited neurite growth on axonin-1, but not on other substrata. In soluble form, axonin-1 had an anti-adhesive effect, as revealed by perturbation of neurite fasciculation. In view of their structural similarity, we conclude that secreted and membrane-bound axonin-1 interact with the same growth-promoting neurite receptor. The fact that secreted axonin-1 is functionally active, together with our previous findings that it is secreted from an internal cellular pool, suggests a functional dualism between membrane-bound and secreted axonin-1 at the site of secretion, which is most likely the growth cone. The secretion of adhesion molecules could represent a powerful and rapidly acting regulatory element of growth cone–neurite interactions in the control of neurite elongation, pathway selection, and possibly target recognition.

Materials and Methods

Materials

Secreted axonin-1 was purified as previously reported (Ruegg et al., 1989b); and it was shown to be free of contaminating cell adhesion molecules. L(G4) was isolated by immunoaffinity chromatography, using a monoclonal anti-G4 antibody provided by Dr. Fritz Rathjen (Zentrum für Molekulare Neurobiologie, Universität Hamburg, Hamburg, Germany) (Rathjen et al., 1987a). Laminin was purchased from GIBCO-BRL Laboratories (Gaithersburg, MD). BSA was from Miles Scientific (Naperville, IN).
Antibodies against axonin-1 were raised in goat as previously described (Ruegg et al., 1989a). IgG was purified by affinity chromatography on protein G Sepharose. IgG was subjected to proteolytic digestion with papain (Porter, 1959). FC fragments were removed by passage over protein G Sepharose and the Fab fragments were further purified by cation exchange chromatography (Mono S, Pharmacia Fine Chemicals, Uppsala, Sweden).

Cell Cultures

For the culture of dissociated DRG neurons, axonin-1 and the other substrata Li(G4), laminin, and BSA were adsorbed directly to tissue culture plastic and the cells were cultivated in defined, serum-free medium. With intact ganglia, the conditions previously reported by Lagenaur and Lemmon (1987) yielded better attachment and survival of the ganglia, and hence, the substrata were coated onto a film of nitrocellulose and ganglia were maintained in medium containing FCS. For coating, circular areas (50 mm²) of culture plates were covered with 20 μl axonin-1, Li(G4), laminin, or BSA, respectively, at a concentration of 80 μg/ml in PBS, and incubated for 2 h. The plates were washed twice with PBS and blocked with 10 mg/ml BSA in PBS for 45 min.

DRGs were dissected from 10-d-old chicken embryos and dissociated (Sonderegger et al., 1985). The serum-free culture medium for the dissociated cells was composed of Eagle's minimal essential medium in Earle's salts, 5 mg/ml BSA, 100 μg/ml transferrin, 20 ng/ml nerve growth factor, 10 μg/ml insulin, 20 ng/ml triiodothyronine, 40 nM progesterone, 200 ng/ml corticosterone, 200 μM putrescine, and 60 mM sodium selenite. The medium for undissociated ganglia was composed of minimal essential medium with 10% FCS and 20 ng/ml nerve growth factor. When Fab fragments of goat IgG were added, concentrations were 250 μg/ml for anti-axonin-1 Fab fragments and 500 μg/ml for nonimmune Fab fragments.

Fasciculation perturbation experiments with soluble axonin-1 were carried out with intact embryonic DRGs cultured on collagen in serum-free medium containing ovalbumin at a concentration of 5 mg/ml. Soluble axonin-1 was added to a final concentration of 320 μg/ml. In control cultures, axonin-1 was substituted by an equal amount of ovalbumin or by a corresponding volume of PBS. After incubation for 40 h, the cultures were fixed with 2% glutaraldehyde in 50 mM cacodylate, pH 7.2. For light microscopic inspection, an inverted microscope equipped with phase contrast optics was used. Specimens for scanning EM were prepared by critical point drying and gold sputter coating.

Measurements of Neurite Lengths

Dissociated DRG cells were cultured in triplicates on different substrata for 20 h in defined, serum-free medium. Neurite growth was quantified on an inverted microscope using phase contrast optics. The plating area inspected was ~20 mm² for axonin-1, Li(G4), and laminin, and 80 mm² for BSA. A neurite was defined as a process extending from the neuronal cell body by more than a cell diameter. Only neurites that emerged from an isolated neuron (not a clump of cells), and did not contact other neurites or cells, were included for length determination. Neurite length of the longest branch was measured along the neurite from the cell soma to the most remote tip. For total length all neuritic branches elaborated by a neuron were measured.

For the presentation of the distribution of the neurite lengths developed by the neurons cultivated on a given substratum, the graphic representation introduced by Chang et al. (1987) was adopted. The percentage of neurites with lengths shorter than a given length was plotted versus neurite length taken either as the length of the longest neurite branch or the total length of the neuritic tree developed per neuron. As a characteristic for the neurite growth promoting potency, the neurite length developed by 50% of the neuritic tree was determined according to Bradford (1976). For immunoblotting, the procedure elaborated by Towbin et al. (1979) was adopted; immunodetection of antigens was performed as described by Hawkes et al. (1982), using peroxidase-conjugated second antibody. Goat anti-axonin-1 serum was used at a dilution of 1:1,000. Rabbit IgG against Li(G4), F11, neurofascin, and N-CAM (provided by Dr. F. Rathjen) were used at a concentration of 25 μg/ml.

Results

Promotion of Neurite Growth on Immobilized Secreted Axonin-1

The neurite growth-promoting activity of immobilized axonin-1 was studied in vitro with both intact DRGs and dissociated DRG neurons (Fig. 1). As a comparison, two established neurite growth-promoting substrata were included in this study; namely the basement membrane glycoprotein laminin (Baron-Van Evercooren, 1982) and the AxCAM L1 (Lagenaur and Lemmon, 1987) (which in the chicken is represented by G4 (Rathjen et al., 1987a), SD9 (Lemmon and McLoon, 1986), and possibly Ng-CAM (Friedlander et al., 1986)). As demonstrated in Fig. 1 a, axonin-1 was found to be a very effective promoter of neurite growth from embryonic DRG neurons. Neurites grown from intact DRGs were joined to relatively straight fascicles and virtually no nonneuronal cells had evaded from the ganglia. A comparison with Li(G4) and laminin revealed marked differences in both the neurite growth pattern and the behavior of nonneuronal cells. On Li(G4), in accordance with previous reports (Lagenaur and Lemmon, 1987), neurites of undissociated ganglia were virtually unfasciculated. Laminin was a good substrate for neurite extension; however, in marked contrast to both axonin-1 and Li(G4) it also supported the evasion of nonneuronal cells from the ganglia. Neurites of single neurons differed on the substrata investigated with respect to straightness of growth and degree of bifurcation. Whereas neurites grew straight and rarely developed bifurcations on laminin, they were crooked and highly bifurcated on Li(G4). Neurites on axonin-1 were intermediate to those on laminin and Li(G4) with respect to both straightness of growth and degree of bifurcation (not quantified).

Quantification of Neurite Growth on Axonin-1, Li(G4), and Laminin

A quantitative assessment of neurite growth was carried out on dissociated DRG cultures grown in serum-free medium (Table I and Fig. 2). The percentage of neurons extending neurites was similar on axonin-1, Li(G4), and laminin (Table I). Compared with laminin, the formation of more than one neurite was twice as frequent on axonin-1 and Li(G4). This observation, which may be related to the substratum's adhesiveness for growth cones (for a discussion see Bray, 1987), is in line with the observed degree of neurite bifurcation, where an increasing order from laminin, via axonin-1, to Li(G4) was found. With respect to the mean lengths of the longest neurite branch, cultures on axonin-1 were very similar to those on Li(G4) and laminin. The most extended neuritic trees were significantly larger on axonin-1 and Li(G4) than on laminin. This may be explained by the higher degree of neurite bifurcation on axonin-1 and Li(G4).

The distribution of neurite lengths on axonin-1, Li(G4), laminin, and BSA are given in Fig. 2. Adopting the graphic
representation recently introduced by Chang et al. (1987), the percentage of neurons with neurites longer than a given length was plotted against the neurite length. The length distribution of the longest neurites (solid line in Fig. 2) were similar on axonin-1 and laminin, and the neurite lengths elaborated by 50% of the neurons (NL50) on these substrata were 110 and 125 μm, respectively. Neurites on LI(G4) were slightly longer, the NL50 being 150 μm. The total length of the neuritic tree per neuron (dashed line in Fig. 2) on axonin-1 was shorter than on LI(G4), but longer than on laminin. Corresponding NL50 values were 300 μm on LI(G4), 210 μm on axonin-1, and 170 μm on laminin, respectively.

Antibody Perturbation of Neurite Growth on Axonin-1

The specificity of the neurite growth-promoting effect of...
axonin-1 was investigated by the cultivation of dissociated DRG neurons in serum-free medium in the presence of axonin-1-specific monovalent antibodies. As shown in Fig. 3, Fab fragments of polyclonal goat IgG against axonin-1 at a concentration of 250 μg/ml virtually eliminated neurite extension on axonin-1. Although used at double concentration (500 μg/ml), nonimmune Fab fragments of goat IgG did not interfere with neurite growth. Neurite extension on Li(G4) and laminin was not affected by anti-axonin-1 Fab fragments.

**Screening of AxCAMs for Soluble Extracellular Forms**

Extracellular humors of the central nervous system were screened by immunoblotting for the presence of soluble forms of four AxCAMs of the chicken; namely Li(G4), F11, neurofascin, and axonin-1. N-CAM, although not an AxCAM, was included because of its expression in axonal membranes (Fig. 4). 7.5 μg of vitreous humor proteins of E14 chicken embryos were analyzed. To estimate the concentration relative to vitreous humor, a volume-matched quantity of cerebrospinal fluid was chosen. As a comparison, 7.5 μg of proteins from "high pH-stripped" brain membranes were included. With these amounts of membrane proteins, axonin-1 immunoreactivity was only in a faint band, whereas all other AxCAMs were strongly represented by bands of considerable intensity and thickness. N-CAM immunoreactivity, as expected, was spread between 90 and over 200 kD. Among the proteins of the cerebrospinal and the vitreous fluid, axonin-1 immunoreactivity was found in a strong band. As judged from the intensity of the immunoreactive band, the concentration of axonin-1 was somewhat higher in the vitreous than in the cerebrospinal fluid. At the protein quantities compared, soluble axonin-1 in the vitreous humor of the eye and the cerebrospinal fluid predominated; the membrane-bound form was minor. This distribution is in line with the distribution between soluble axonin-1 of the vitreous fluid of the eye and tissue-associated axonin-1 of the retina, as determined by ELISA at embryonic day 14, where a 30-fold excess of soluble axonin-1 was found (Ruegg et al., 1989b). Li(G4), F11, and N-CAM occurred predominantly in the membrane fraction, but minor amounts were also present in the vitreous humor and the cerebrospinal fluid. Li(G4) was more prominent in the cerebrospinal fluid, whereas F11 had a higher concentration in the vitreous humor. Neurofascin, as Li(G4) and F11 had a major band at ~130 kD in the membrane fraction. The immunoreactive band pattern in solution differed markedly between the vitreous humor and the cerebrospinal fluid. A relatively weak band of 125 kD was found in the vitreous humor and the cerebrospinal fluid. The immunoreactive band pattern in solution differed markedly between the vitreous humor and the cerebrospinal fluid. A relatively weak band of 125 kD was found in the vitreous humor. In the cerebrospinal fluid a large number of bands between 55 and 185 kD were found, the apparent molecular weights of the major bands closely corresponding to the neurofascinin-1 immunoreactive peptides that were described by Rathjen et al. (1987b) and are thought to be proteolytic fragments of the
Figure 4. Comparison of soluble and membrane-bound forms of axon-associated adhesion molecules. Proteins of vitreous humor (V), cerebrospinal fluid (C), and "stripped" brain membranes (M), were subjected to SDS-PAGE, and electrotransferred to nitrocellulose membrane. For immunodetection, nitrocellulose membranes were incubated with polyclonal antibodies against axonin-I (Ax), Li(G4), F11, neurofascin (Nf), and N-CAM (NCAM), as well as with nonimmune serum. For visualization, peroxidase-conjugated secondary antibody was used. The mounts of proteins applied to the gel were matched between the membrane fraction and the vitreous humor sample (7.5 µg protein/lane). To obtain a comparison of the relative concentration in the extracellular fluids of the nervous system, the sample of the cerebrospinal fluid proteins was matched to the vitreous humor protein sample by volume (7.5 µl or 24 µg protein/lane).

185-kD form. N-CAM was found in trace amounts in both the vitreous humor and the cerebrospinal fluid.

These data illustrate that the occurrence of cell adhesion molecules in soluble form in the extracellular humors of the brain is not an uncommon finding. However, axonin-I is conspicuous by the fact that its soluble form quantitatively out-ranks the membrane-bound form. In this respect, axonin-I is clearly distinct from Li(G4), F11, neurofascin, and N-CAM, which have their predominant fraction or at least a substantial proportion of the total immunoreactive material in the membrane-associated form.

Perturbation of Neurite Fasciculation by Soluble Axonin-I

The effect of soluble axonin-I on axonal interactions was tested in a fasciculation perturbation assay on cultures of intact DRGs on a collagen substratum. As demonstrated in Fig. 5, the presence of soluble axonin-I in the culture medium had a striking effect on the growth pattern of the emerging neurites. Whereas the neurites formed thick bundles in the absence of axonin-I, virtually no fasciculation occurred when soluble axonin-I (320 µg/ml) was present in the culture medium. Similar to previously reported perturbation experiments with anti-axonin-I Fab fragments (Ruegg et al., 1989b), neurites grown in the presence of soluble axonin-I developed a dense meshwork. As the most straightforward interpretation, this phenomenon reflects a reduced tendency of axons to stick together due to the action of cell adhesion molecules, leading to a relative preference for the substratum.

Discussion

The soluble, axonally secreted form of the axon-associated cell adhesion molecule axonin-1 was found to be a strong promoter of neurite growth when offered as a substratum to cultured sensory neurons, and to perturb fasciculation when present in soluble form during neurite growth. In accordance with the generally accepted model, that AxCAMs exert their adhesive and neurite growth-promoting function by the binding of their membrane surface-exposed domain to appropriate receptors on adjacent neuritic membranes (Rutishauser and Jessell, 1988), the neurite growth-promoting function of immobilized axonin-I is interpreted to result from interaction with a neuritic adhesion receptor. In view of the presence of membrane-bound axonin-I on fasciculated axons during neural development and the high degree of structural similarity between soluble and membrane-bound axonin-I (Ruegg et al., 1989b), it appears likely that both forms of axonin-I recognize the same receptor site.

Dual localization in brain membranes and extracellular humors of the central nervous system was shown to be a feature that is not unique to axonin-I. In accordance with previous observations on the release of Li(NILE) (Salton et al., 1983; Stallcup et al., 1983; Sweadner, 1983) and Tag-1 (Furley et al., 1990) from cultured rat neurons, we found that other major AxCAMs of the chicken were also present in substantial quantities in both the vitreous humor and the cerebrospinal fluid. However, the pronounced quantitative predominance of the soluble form makes axonin-I especially intriguing with respect to considerations on the functional role of released AxCAMs. In principle, release of an AxCAM could occur either after having exerted a function in the membrane-bound form at the axonal surface or be targeted for some function in the extracellular space. That secreted axonin-I is not exposed at the cell surface before release, but originates directly from an intracellular pool (Ruegg et al., 1989b) favors the hypothesis that it is secreted to fulfill a role as a soluble molecule, rather than being released after having fulfilled a function at the cell surface. Functional roles envisaged for released forms of other adhe-
Figure 5. Perturbation of neurite fasciculation by soluble axonin-1. Chicken embryonic dorsal root ganglia were cultured on collagen in serum-free medium containing 5 mg/ml ovalbumin. Axonin-1 was added to a final concentration of 320 μg/ml. For controls, axonin-1 was substituted with 320 μg/ml ovalbumin or a corresponding volume of PBS. After incubation for 40 h, the cultures were fixed and subjected to light microscopic inspection with phase contrast optics (a and c), or scanning EM (b and d). (a and b) Neurites grown in the absence of axonin-1 (5.32 mg/ml ovalbumin); (c and d) Neurites grown in the presence of axonin-1 (5 mg/ml ovalbumin + 0.32 mg/ml axonin-1). Bars, 100 μm.

Expression molecules such as fibronectin (Yamada and Kennedy, 1984), cell CAM 120/80 (Wheelcock et al., 1987), and N-CAM (Bock et al., 1987; Edelman et al., 1987; Crower et al., 1988) include both the inhibition of cell adhesion in soluble form and adhesion-promoting activity when attached to the cell membrane or the extracellular matrix; for fibronectin (Yamada and Kennedy, 1984; Johansson and Höök, 1984) and cell CAM 120/80 (Wheelcock et al., 1987), the soluble form has been reported to reduce the cell adhesive function of its membrane-bound counterpart in vitro, and a role in vivo as an "autogenous inhibitor" of cell adhesion has been suggested. Thus, as a test for a possible anti-adhesive function, the effect of soluble axonin-1 on the fasciculation pattern of dorsal root ganglia neurites was investigated. Indeed, soluble axonin-1 added at a concentration of ~2.5 × 10⁻⁶ M (320 μg/ml) to growing neurites in vitro reduced fasciculation strongly, a phenomenon most likely due to reduced interaxonal adhesion. Whether soluble axonin-1 in the extracellular space of the central nervous system reaches concentrations high enough for an anti-adhesive function to become effective in vivo remains to be determined. In the vitreous cavity of the eye, which is contiguous with the extracellular space of the retina and is a diffusional sink for proteins secreted from retinal neurons, secreted axonin-1 was measured to be present at ~5 × 10⁻⁸ M (Ruegg et al., 1989b), a concentration clearly not effective for defasciculation in vitro. However, it is conceivable that the concentrations in proximity to the sites of secretion into the narrow extracellular space of the retina are substantially higher and reach a level sufficient to exhibit anti-adhesive activity.

Modulation of adhesive membrane contact by receptor saturation could have advantages over other regulatory prin-
principles such as downregulation of corresponding membrane proteins. Secreted axonin-1 is derived from a biosynthetic pool with a $t_{\alpha}$ of $\sim 5$ h, whereas the pool giving rise to membrane-bound axonin-1 has a $t_{\alpha}$ of $>100$ h (Ruegg et al., 1989b). Hence, a modulation of the axonin-1-dependent adhesive forces could be achieved considerably faster by receptor saturation with secreted axonin-1 than by shutting off the synthesis of membrane-bound axonin-1. Furthermore, the site of axonal secretion is thought to be virtually restricted to the axon endings (Kelly, 1988). If this holds also for the secretion of axonin-1, then the highest local concentrations may be expected in the extracellular space surrounding the secreting growth cones. Conceivably, by competing with membrane-bound axonin-1 for a common receptor, axonally secreted axonin-1 could subvert the adhesive situation at the growth cone moving along an axon pathway in favor of other adhesion molecules (Rutishauser and Jessell, 1988), and hence represent a determinant for the growth cones' adhesive behavior during the period of axonal growth, pathfinding, and target recognition.

We are indebted to Dr. Fritz Rathjen for antibodies versus LI(G4), F11, neurofascin, and N-CAM. Scanning EM was kindly contributed by Dr. Peter Groscurth.

This work was supported by grants from the Swiss National Science Foundation, the Geigy-Jubiläumsstiftung, the EMDO-Stiftung, the Hartmann Müller-Stiftung, the Sandoz-Stiftung, the Roche Research Foundation, the Schweizerische Multiple Sklerose Gesellschaft, and the Jubiläumsstiftung der Schweizerischen Lebensversicherungs- und Rentenanstalt für Volksgesundheit und medizinische Forschung.

Received for publication 21 June 1990 and in revised form 16 October 1990.

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