A Soluble Form of the F3 Neuronal Cell Adhesion Molecule Promotes Neurite Outgrowth

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Abstract. The F3 molecule is a member of the immunoglobulin superfamily anchored to membranes by a glycane-phosphatidylinositol, and is predominantly expressed on subsets of axons of the central and peripheral nervous system. In a previous paper (Gennarini, G., P. Durbec, A. Boned, G. Rougon, and C. Goridis. 1991. *Neuron.* 6:595–606), we have established that F3 fulfills the operational definition of a cell adhesion molecule and that it stimulates neurite outgrowth when presented to sensory neurons as a surface component of transfected CHO cells. In the present study the question as to whether soluble forms of F3 would be functionally active was addressed in vitro on cultures of mouse dorsal root ganglion neurons. We observed that preparations enriched in soluble F3 had no effect on neuron attachment but enhanced neurite initiation and neurite outgrowth in a dose-dependent manner. By contrast, soluble NCAM-120 does not have any measurable effect on these phenomena. Addition of anti-F3 monovalent antibodies reduced the number of process-bearing neurons and the neuritic output per neuron to control values. Addition of cerebrospinal fluid, a natural source of soluble F3, also stimulated neurite extension, and this effect was partially blocked by anti-F3 antibodies. Our results suggest that the soluble forms of adhesive proteins with neurite outgrowth-promoting properties could act at a distance from their site of release in a way reminiscent of growth and trophic factors.

THE directional growth of axons is one of the first cellular events leading to the differentiation of the neuronal cell. It involves multiple interactions between axons and their environment made up of other axons, neighboring cells, and extracellular matrix (Dodd and Jessell, 1988). The molecular basis of pathway selection by developing axons is poorly understood. Growth cones seem to be endowed with surface receptors that sense local cues and are coupled to effector systems which induce activities such as elongation, branching, turning, retraction, and finally synapse formation. The ligands that bind to these receptors may be soluble molecules such as growth and trophic factors or adhesive molecules either on neighboring cell surfaces or in the extracellular matrix (Letourneau, 1987).

In recent years, knowledge of the structure and function of neuronal surface proteins which mediate direct cell–cell or cell–substrate interactions has grown considerably. Many of them are members of one of three structural families: the cadherins (Takeichi, 1988), the integrins (Ruoslahti, 1988), and the immunoglobulin superfamily (Williams and Barclay, 1988). Several glycoproteins that are localized predominantly on the axons of developing vertebrate neurons have been characterized immunochemically (Rathjen and Schachner, 1984; Rathjen et al., 1987*a*,*b*), cloned and sequenced (Moos et al., 1988; Brümmendorf et al., 1989; Furley et al., 1990; Gennarini et al., 1989*a*,*b*). They belong to the immunoglobulin superfamily and share common structural features, in that each of these molecules contains six NH_2-terminal immunoglobulin-like domains and sequences resembling fibronectin type III repeats in their premembrane regions. Among them, only L1 exhibits a membrane-spanning region (Moos et al., 1988) whereas rat TAG-1 (Furley et al., 1990), chicken F11 (Brümmendorf et al., 1989) and mouse F3 (Gennarini et al., 1989*a*) have a glycosylphosphatidylinositol (GPI)[1] membrane anchor. The dynamic changes in their expression patterns and their localization to certain neuronal cell bodies and fibers during development are consistent with the hypothesis that they are important regulators of axonal growth, fasciculation, and synaptogenesis. Anti-L1 antibodies (Abs) reduce neurite extension on other neurites (Chang et al., 1987). Anti-F11 Abs have defasciculating properties (Rathjen et al., 1987*b*) and also disturb the elongation of neurites on other neurites (Chang et al., 1987). TAG-1 has been directly demonstrated to promote neurite growth when used as a substrate (Furley et al., 1990).

1. *Abbreviations used in this paper*: Ab, antibody; CSF, cerebrospinal fluid; DRG, dorsal root ganglia; GPI, glycosylphosphatidylinositol; NCAM, neural cell adhesion molecule; PI-PLC, phosphatidylinositol-specific phospholipase C.

In an earlier study (Gennarini et al., 1991), we transfected F3 cDNA into CHO cells and established that, in the isolated transfectants, the cDNA codes for a 142-kD GPI-anchored protein. F3-expressing transfectants, when used as a culture substrate for sensory neurons, showed a markedly enhanced ability to promote neurite outgrowth as compared with nontransfected cells. The observed effect could be blocked by monovalent anti-F3 Abs raised against the immunoglobulin domain region of the protein.

One of the features of most GPI-anchored molecules is that they are found either membrane bound or in soluble form spontaneously released by the cells. This was also the case of F3 molecules expressed by the transfectants we used in our assay. This observation raises questions as to the possible role of released F3 in our neurite outgrowth assay. In this study, we addressed this question by investigating the effect of preparations enriched in soluble F3 on the pattern of neurite outgrowth. Our results demonstrate that F3 contained in such a preparation is a strong promoter of neurite initiation and neurite extension by dorsal root ganglia (DRG) neurons, and hence is functionally competent when presented in soluble form.

Materials and Methods

Cell Culture and Cerebrospinal Fluid Collection

The CHO cell line LR-73 (Pollard and Stanners, 1979) and the F3-transfected CHO cell lines E12 and 1A (Gennarini et al., 1991) were maintained in alpha-MEM supplemented with 10% FCS and 50 IU/ml penicillin, 50 μg/ml streptomycin. Cerebrospinal fluid (CSF) was collected by spinal puncture using a 5-μl glass capillary (CML, Nemours, France) on frozen CO_2-anesthesized animals, which were subsequently used for dissecting the DRGs. After collection, the pooled CSF was centrifuged at 1,000 *g* for 10 min before dilution in the culture medium. DRGs were dissected out from newborn (P0) mice and dissociated as described by Rougon et al. (1983) and Gennarini et al. (1991). The cells were plated in 24-well tissue culture plates (Nunc, Poly Labo, Strasbourg, France) treated with poly-L-lysine (2 μg/ml), in DME supplemented with 10% FCS, antibiotics, and 50 μg/ml nerve growth factor. Culture media were supplied by Gibco-BRL (Cergy-Pontoise, France). For experiments designed to test the effects of soluble F3, cells were plated in $F3^+$ or $F3^-$ supernatants obtained as described below. In some experiments, dilutions of $F3^+$ or $F3^-$ supernatants or of CSF in the above medium were used. Some assays were conducted in the presence of 200 μg/ml Fab' fragments of anti-F3 or neural cell adhesion molecule (NCAM) Abs added to the culture medium at the time of plating.

Antibodies

For immunodotting detection of F3, the first generation polyclonal Ab prepared as described was used (Gennarini et al., 1989*b*). For immunoblotting and functional assays, a rabbit polyclonal Ab prepared against a fusion protein comprising the F3 Ig-like domains was used (Gennarini et al., 1989*a*). A site-directed rabbit polyclonal anti-NCAM Ab recognizing the NH_2-terminal domain of the molecule was used for immunoblotting experiments (Rougon and Marshak, 1986). To monitor NCAM effects in tissue culture, the Fab' fragments were prepared from a rabbit antiserum raised against immunopurified adult mouse NCAM (Gennarini et al., 1986).

Antilaminin rabbit polyclonal Ab was a kind gift from Dr. J. C. Lissitsky (Centre National de la Recherche Scientifique, Marseille).

Preparation of F3-enriched Supernatant

Parental or transfected CHO cells were harvested in Versene (Gibco-BRL, Cergy-Pontoise), pelleted and resuspended at 10^6 cells/ml in DME. They were treated for 30 min at 37°C with phosphatidylinositol-specific phospholipase C (PI-PLC) enzyme purified in our laboratory (Thèveniau et al., 1990) from *Bacillus thuringiensis* and used at 0.05 IU/ml. Supernatants were recovered and centrifuged at 100,000 *g* for 1 h. The enzyme was removed by two successive runs of incubation of the supernatants (4 h at 4°C under shaking) with anti-PI-PLC polyclonal Ab coupled to Sepharose beads (Thèveniau et al., 1990). Supernatants were then filtered, supplemented with 10% FCS, antibiotics and 50-μg/ml nerve growth factor, (Sigma Chemical Co., La Verpillière, France) and immediately used for subsequent assays.

Tissue Extracts and Immunoblot Detection

Tissue extracts were prepared as previously described (Rougon et al., 1982). Briefly, tissues were homogenized in 10 mM Tris-HCl, pH 7.8, containing 2% (wt/vol) NP-40, 1 mM EDTA, and protease inhibitors. The extracts were centrifuged at 100,000 *g* for 1 h at 4°C. The supernatants were collected and protein concentration was determined (Duley and Grieves, 1975). The protein concentration was adjusted to 3 mg/ml; samples were boiled for 3 min in reducing electrophoresis buffer. Aliquots containing equivalent amounts (30 μg) of protein were then resolved by 7.5% SDS-PAGE. For CSF, 10-μl aliquots were centrifuged and the supernatants were boiled in sample buffer as above. As a control for CSF experiments, normal mouse serum was processed in the same way. After electrophoretic separation, proteins were transferred to nitrocellulose sheets (Nitroscreen West NEN Products, Les Ulis, France) for 2 h at 0.6 A. After a 2 h saturation in PBS containing 5% skimmed milk (wt/vol), the sheets were probed with primary Abs, 1:1,000 dilution for anti-NCAM (Rougon and Marshak, 1986) and 1:250 for anti-F3 (Gennarini et al., 1989*a*) polyclonal Abs for 15 h at 4°C under shaking. Bound Abs were revealed by reaction with iodinated protein A (0.5×10^6 cpm/ml) and subsequent autoradiography.

Immunodotting Procedure

Twofold serial dilutions of serum-free supernatant either containing ($F3^+$) or not containing ($F3^-$) the F3 molecule were made in PBS. 2 μl of each dilution was spotted onto nitrocellulose. After saturation with PBS-milk the sheets were reacted with anti-F3 and anti-NCAM polyclonal Abs and treated as described for immunoblots.

Coating Assays

To test the ability of soluble F3 to adhere to culture dishes, serial dilutions of F3-containing supernatants or of a laminin solution (1 μg/ml), used as a control, were made in DME either containing or not containing 10% FCS. The dilutions were incubated for 6 h at 37°C in 96-well tissue culture plates pretreated with poly-L-lysine. After three washes with PBS, the plates were incubated for 20 h at 4°C with anti-F3 (1:250 diluted) or antilaminin (1:250 diluted) polyclonal Abs. The wells were washed three times with PBS and bound Abs were revealed by incubation with ^{125}I-protein A (0.5×10^6 cpm/ml). After washing, the wells were rinsed twice with 50 μl 10% SDS and the pooled fractions then counted in a γ counter. To estimate nonspecific binding, controls were done either without laminin or F3-containing solutions or by omitting the addition of Abs.

Morphometric Analysis

Every 4 h, after plating, the cells in each well were examined under a microscope (ICM 405; Carl Zeiss, Inc., Thornwood, NY). Neurons showing neurites longer than the diameter of the cell body were counted. More than 100 neurons were counted for each individual experimental condition. For subsequent analysis wells were gently rinsed with medium without FCS and fixed with 4% paraformaldehyde.

To measure total neurite length and cell surface area in short-term cultures, camera lucida drawings of neurons visualized by phase-contrast microscopy were analyzed using an Apple Scanner digitizing tablet and an Image 1.29 program written for Mac II software. In all morphometric studies, only isolated neurons, i.e., neurons whose cell bodies were at least 200 μm from the soma of their nearest neighbor and whose neurites were not in contact with neighboring cell neurites, were analyzed. Only processes whose length was greater than the diameter of the cell body were scored. More than 200 randomly chosen neurons (~40 neurons from 11 separate experiments, each conducted with separately prepared batches of supernatants [5] or CSF [6] and different dissections of DRG) were evaluated for each experimental condition. For Fab inhibition experiments five evaluations were done: two for supernatants and three for CSF. Data are expressed as the mean ± SEM.

The significance of the difference in the distribution and neuritic network density in different experimental conditions was calculated using Duncan's statistical test (Duncan, 1955).

Results

Preparation of F3 in Soluble Form

As reported for other GPI-anchored molecules, F3 exists in a membrane-bound and a soluble form. F3 was originally purified as a soluble molecule from the medium of forebrain explants (Gennarini et al., 1989*b*). Later on, F3 in mouse brain homogenates was found to be distributed between a buffer-soluble and a membrane fraction (Gennarini et al., 1989*a*). CHO cells, expressing F3 after cDNA transfection, bear the protein at the cell surface, but also release it spontaneously into the culture medium (Gennarini et al., 1991). To search for a role for soluble F3, we prepared culture medium enriched in such molecules. To do so, the highly F3-expressing CHO cell line E12 was treated with PI-PLC as described in Materials and Methods to cleave GPI-anchored molecules of the cell surface. The supernatants were recovered and used as a source of soluble F3 after removal of PI-PLC from the medium. As a control, PI-PLC supernatants were prepared from the parental LR-73 line in an identical manner. The supernatants were examined for their content of soluble F3 and another GPI-anchored molecule we found to be expressed by CHO cells, the 120-kD isoform of NCAM (NCAM-120) (Hé et al., 1987). F3 was readily detectable in the PI-PLC supernatant of E12 but not of LR-73 cells. Most of the cell-associated F3 molecules were released by PI-PLC treatment since the amount of F3 recovered with the cells after treatment was very low (Fig. 1 *A*). The soluble form migrated in SDS-PAGE with an apparent molecular mass slightly higher than that of the membrane-anchored form (148 vs. 142 kD), in agreement with observations made for other GPI-anchored molecules (Low and Saltiel, 1988). As already described, the F3 molecule expressed by E12 cells appeared slightly larger (142 kD) than the one present in the brain (135 kD), probably reflecting differences in glycosylation (Gennarini et al., 1991). By immunodot analysis (Fig. 1 *B*), F3 immunoreactivity could be detected in PI-PLC supernatants of E12 cells up to a 1:16 dilution while supernatants from LR-73 cells were entirely negative. By contrast, equivalent amounts of NCAM-120 were recovered from both cell types. Although precise values of the F3 contents cannot be given using these techniques, the quantities of NCAM-120 and F3 contained in E12 cell supernatants appeared to be in the same range.

Effect of Soluble F3 on Neuron Survival and Neurite Initiation

PI-PLC supernatants prepared from either E12 ($F3^+$) or parental CHO cells ($F3^-$) and supplemented with FCS and nerve growth factor were used as culture medium for single cell suspensions of DRG neurons dissected from newborn mice and plated at a density of 500 cells/2-cm^2 well. Standard culture medium (S) was also used as a control.

Cell adhesion, cell survival, and neurite initiation were analyzed after various time intervals in vitro. 2 h after plating, ~80% (80 ± 5%, n = 10 experiments) of the viable DRG neurons present in the cell suspension adhered to the poly-L-lysine–coated plastic substrate whatever the medium

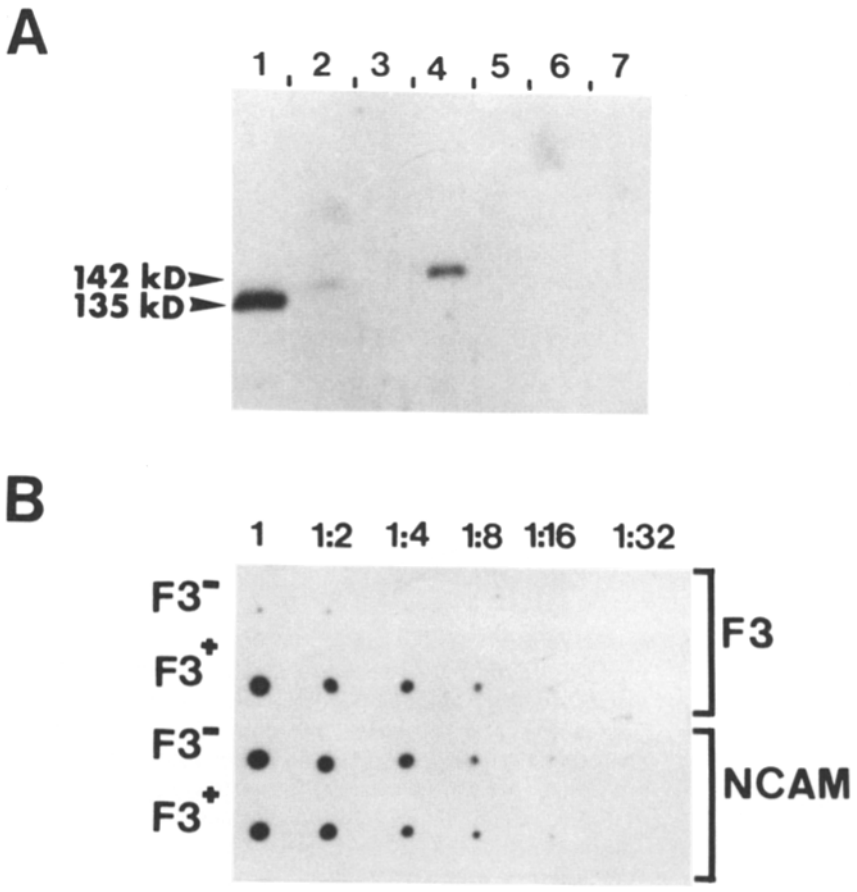

Figure 1. F3 is present in PI-PLC supernatants of E12 but not of LR-73 cells. (*A*) Immunoblot analysis with anti-F3 Abs of the F3 content of 10^6 LR-73 (lane *3*) or E12 (lane *2*) cells (30 μg protein) and the same amount of protein of P28 mouse brain (lane *1*). Lanes *4* and *5* show the amount of soluble F3 molecules recovered after treatment with PI-PLC of 2 × 10^6 E12 and LR-73 cells, respectively. PI-PLC removed the majority of F3 molecules expressed by E12 cells as shown by analysis of E12 (lane *6*) and LR-73 (lane *7*) cells after treatment. (*B*) Immunodot analysis with anti-NCAM and anti-F3 Abs of PI-PLC supernatants. Twofold serial dilutions (2 μl) of supernatants obtained after PI-PLC treatment of 10^7 cells/ml were spotted on nitrocellulose. The sheets were saturated and incubated with the respective Abs. Bound Abs were revealed by incubation with ^{125}I-protein A and subsequent autoradiography. Note that NCAM and F3 immunoreactivity could be detected up to a 1:16 dilution.

used (i.e., F3+, F3−, and S). For each of these conditions, cell survival was determined after 12 and 24 h. Almost all the cells that had adhered to the wells survived during this time *in vitro* in the three culture conditions (74 ± 5%, 77 ± 3%, 73 ± 6% of the cells seeded after 24 h for F3+, F3−, and S medium, respectively).

In the beginning, the cells were round but then they rapidly became flattened and acquired a more spread-out morphology. PI-PLC supernatants had thus no effect on initial survival or adhesion to the substrate. However, a differential response of neurons to F3+ medium became detectable after 8 h in vitro. In cultures grown in F3+ medium some neurons had already started to extend processes, while this was not observed before 12 h in the presence of F3− or standard medium (results not shown).

To analyze the effect of F3+ versus control media on neurite initiation we quantified the number of neurons with neurites after 12 and 24 h of culture. After 12 h in F3+ medium, 40% of the neurons exhibited at least one neurite with a length superior to the size of their cell body as compared with only 10% in the two control media (Fig. 2 *A*). These values reached ~70 and 20–25%, respectively, after 24 h in vitro (Fig. 2 *B*). Hence, F3+ medium promotes neurite initiation of at least 50% of the population of viable neurons. As ~75% of the cells seeded survived over this period whatever the medium used, the effect of F3+ medium on neurite initiation can thus not be attributed to selective survival of a subpopulation of neurons in this condition. The ratios of neurons bearing neurites in F3+ medium to neurons bearing neurites in control media were 4.4 and 2.8 after 12 and 24 h, respectively. This indicates that F3+ medium effect is especially pronounced at early times in culture. In the F3− medium, which contains the same concentration of NCAM-120 as F3+ medium, the number of cells initiating processes was not significantly higher than in standard medium (S) whatever the time after plating.

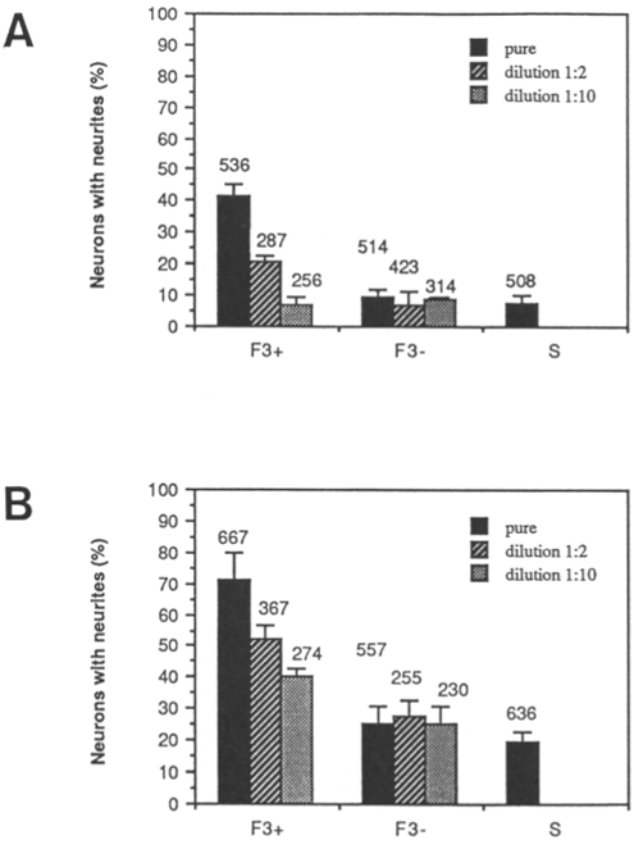

Figure 2. Effect of F3+, F3−, and standard culture media on neurite initiation by DRG neurons. Single cell suspensions of DRG neurons were plated in the indicated dilutions of PI-PLC supernatants obtained after treatment of E12 (*F3+*) and LR-73 (*F3−*) or standard culture medium (*S*) in poly-L-lysine–coated plastic wells. After 12 h (*A*) and 24 h (*B*) in vitro, the cultures were fixed, the number of neurons with at least one neuritic extension longer than one cell body diameter was counted and expressed as the percentage of neurite-bearing neurons over total neurons adhering to the substrate. The data reported are the mean values for five separate experiments conducted with different batches of supernatants and different dissections of DRG neurons from newborn mice. The number of recorded neurons for each condition is indicated above the bars. Bars indicate SEM values.

The neurite outgrowth–promoting effect of F3+ supernatant was clearly dose dependent (Fig. 2); it was highest in the presence of undiluted F3+ medium and not significant after 12 h when the supernatant was diluted 10-fold, although a small effect could still be detected at this dilution after 24 h. By contrast, dilution of the F3− medium did not affect the number of neurite-bearing neurons which remained the same as in standard culture medium. Quantitatively and qualitatively very similar results were obtained using F3+ medium prepared from another F3-transfected CHO clone (clone 1A). As determined by cytofluorometric analysis, this clone expresses around two times more F3 molecules at the cell surface than the clone E12 suggesting that the values recorded with undiluted E12 supernatant are near or at the maximal response (data not shown). Since the only difference between parental and transfected cells should be the expression of F3, these results provide strong evidence that soluble F3 molecules stimulate neurite outgrowth in a dose-dependent manner. Furthermore, they indicate (*a*) that the parental CHO cells neither release endogenous factors nor express GPI-anchored molecules able to influence neurite initiation and, more specifically, (*b*) that soluble NCAM-120 does not have any measurable effect on this phenomenon.

Effect of F3 on Neurite Length and Neuronal Shape

After 24 h in vitro, F3+ medium not only promoted neurite outgrowth but also influenced overall neurite length, and the density of the neuritic network. This is most clearly seen in low magnification phase-contrast micrographs of neurons grown in the three media—S, F3−, and F3+ (Fig. 3, *A′–C′*). At longer durations in vitro (72 h or more), the differences among culture conditions were less obvious since neurites also grew in F3− and S media although apparently at a slower rate (not shown).

We performed quantitative analyses of the average size of the neuritic network per neuron and of the number of neurites extended from the cell body after 24 h in vitro for well-isolated neurons. A plating density of 500 cells/2-cm² well was found to be appropriate to observe a sufficient number of isolated neurons with growing neurites (Fig. 3, *A*, *B*, and *C*), and camera lucida drawings of randomly visualized individual neurons which had grown processes were made in the different culture conditions (Fig. 4). The average neurite

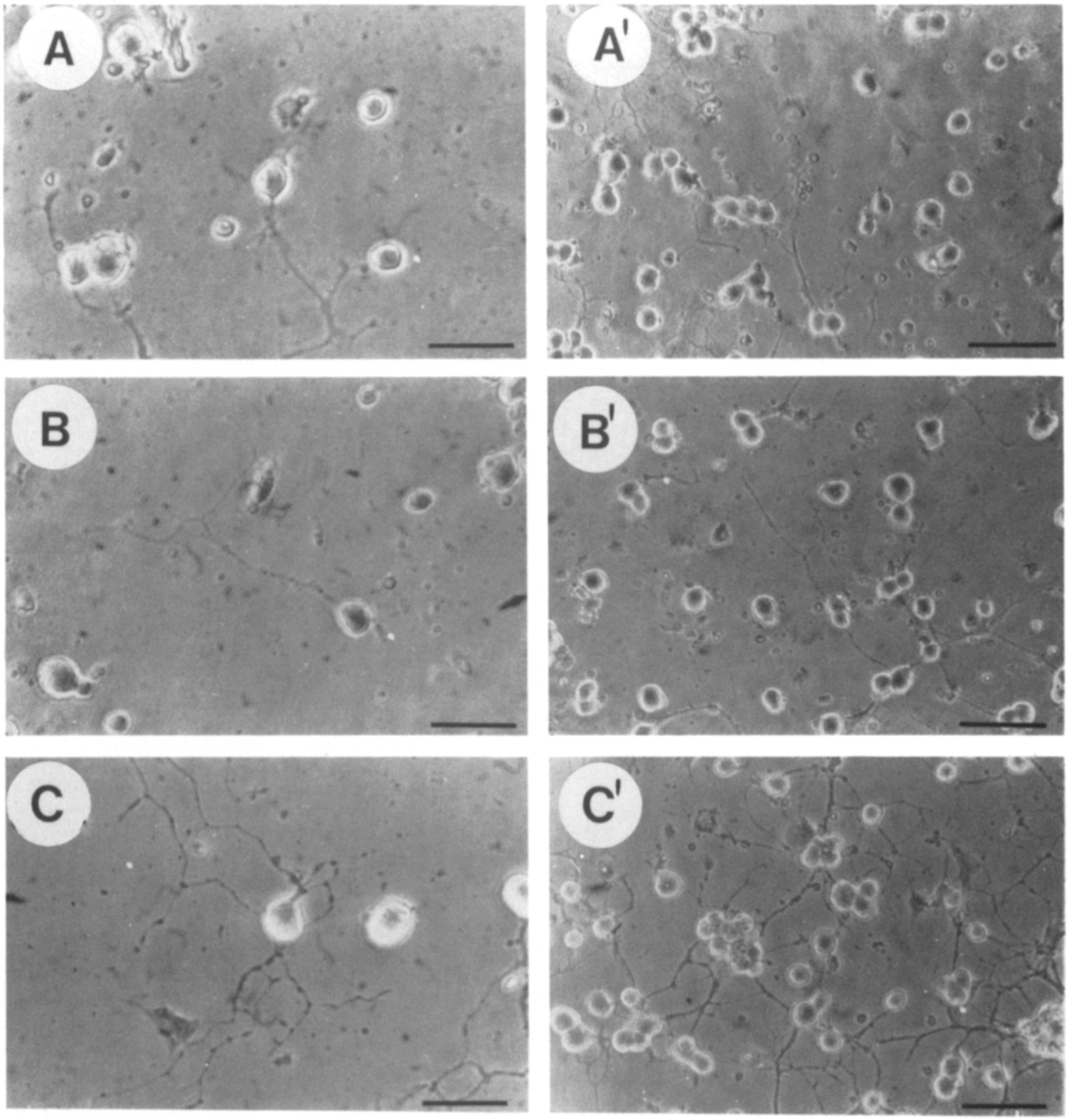

Figure 3. Phase-contrast photomicrographs of DRG neurons grown for 24 h in different media. At that time, 70% of the neurons extended neurites in F3$^+$ medium (*C* and *C'*) compared with 20–25% for neurons in F3$^-$ medium (*B* and *B'*) or standard medium (*A* and *A'*). Representative examples of isolated neurons with processes plated at 500 cells/2-cm^2 well (*A–C*) show that in F3$^+$ medium neuritic length is higher (*C*) than in control media (*A* and *B*). At lower magnification, it can be seen that the neuritic network is denser in F3$^+$ medium. Bars: (*A–C*) 50 μm; (*A'–C'*) 80 μm.

length per neuron was determined in five separate experiments in which different batches of F3$^+$ and F3$^-$ supernatants and different DRG preparations were used. In Fig. 5 *A*, neurite lengths in the different culture conditions were compared in cumulative frequency distribution plots for two representative experiments; Table I summarizes the data and their statistical analysis for all experiments. Clearly, the neurite lengths recorded per neuron are continuously shifted to higher values in the presence of F3$^+$ medium. Although the mean values somewhat vary from one experiment to another, they were always higher for neurons grown in F3$^+$ medium compared with F3$^-$ or standard medium. Statistical analysis of the pooled data gave an average neuritic length in F3$^+$ medium of 944 ± 245 μm (mean ± SEM) compared to 322 ± 59 and 329 ± 100 μm for F3$^-$ and standard medium, respectively. As was observed for neurite initiation, very similar results were obtained when the effect of F3$^+$ medium prepared from clone 1A was analyzed (not shown).

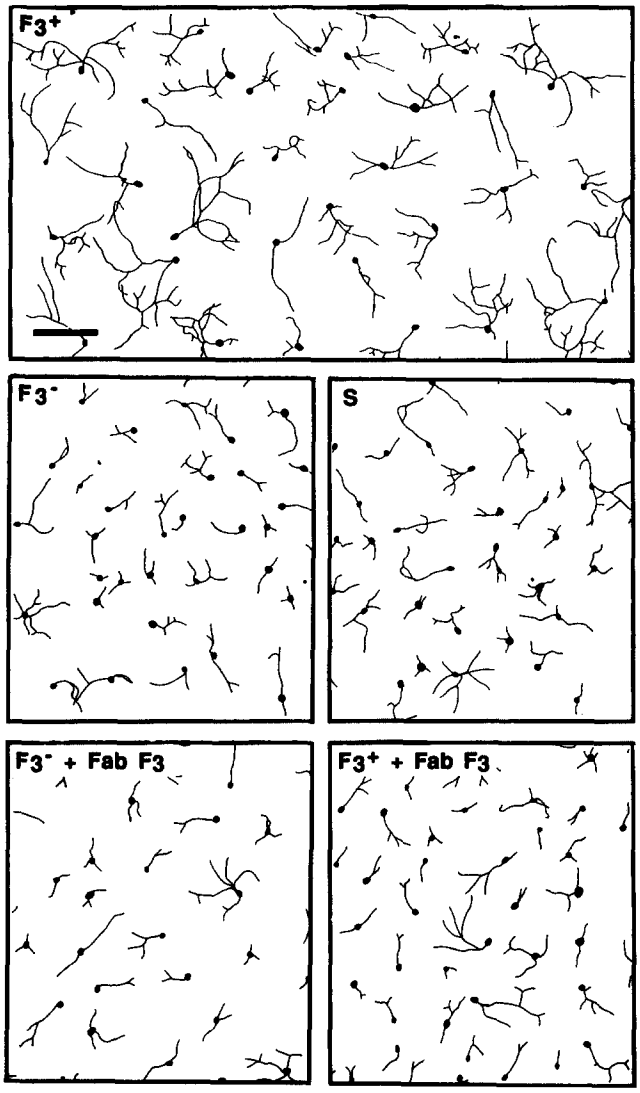

Figure 4. Morphological analysis of isolated DRG neurons after 24 h in different media. Neurons plated at a density of 500 cells/2-cm² well were fixed with paraformaldehyde and stained with Coomassie blue. The first isolated 40-process-bearing cells observed were drawn with the help of a light chamber. The top shows such neurons cultured in F3⁺ medium; the middle panels represent neurons grown in F3⁻ and standard medium (*S*), respectively. On the bottom, experiments were conducted in the continuous presence of 200 μg/ml of Fab′ fragments of anti-F3 Abs. Note that this treatment considerably reduced the size of the neuritic trees when neurons were grown in F3⁺ medium (*F3⁺ + Fab F3*). Bar, 300 μm.

To ascertain that the observed effects were indeed mediated by F3 present in the F3⁺ medium, two experiments were done in the continuous presence of Fab′ fragments of anti-F3 Abs raised against the immunoglobulin domains of the molecule (Gennarini et al., 1989*a*), or of Fab′ fragments of anti-NCAM Abs used as a control. Addition of anti-F3 Abs reduced average neurite length to a level very similar to the one observed for control media (Fig. 4 and Table I). The cumulative frequency plot data (Fig. 5 *A*) confirm that anti-F3 Abs completely abolish the effect of F3⁺ media. They further show that the populations of neurite lengths measured in F3⁻ medium in the presence or absence of the anti-F3 Fabs are almost superimposable, indicating that the Abs at the concentrations used have no toxic or other nonspecific effects. Hence, DRG neurons respond to F3 and not to other molecules in the F3⁺ medium and, as already reported (Gennarini et al., 1991), F3 stimulates neurite outgrowth via epitopes located within the immunoglobulin domains of the molecule. By contrast, anti-NCAM Abs were without effect on the studied parameters, in agreement with reported data (Chang et al., 1987) showing that anti-NCAM Abs do not affect neuritic outgrowth of chick sympathetic neurons.

Cell body sizes of isolated neurons were measured for the different media and found to be 21.2 ± 2 μm (F3⁺), 21.0 ± 2 μm (F3⁻), and 20.9 ± 2 μm (S). The homogeneity of the sizes measured in the different media indicates that, at least as far as cell size is concerned, the same neuronal populations were examined.

We then compared the effects of the different media on the number of primary neurites (neurites directly attached to the cell body) extended by individual neurons (Fig. 5 *B*). The small increase in the fraction of neurons bearing two primary processes at the expense of one-neurite-bearing cells recorded in the presence of F3⁺ medium did not reach statistical significance. Clearly then, F3 has a much more pronounced effect on the proportion of neurons that do extend neurites and on the length of neurites than on the number of neurites initiated per neuron. No differences were observed for cells cultured either in standard or F3⁻ medium.

The Soluble Form of F3 Is the Active Form

Although F3 was present in our supernatants in soluble form as the cleavage product of PI-PLC treatment of E12 cells, the data presented above do not exclude that the observed effects were in fact due to the fraction of the molecules that would adhere to poly-L-lysine–coated plastic wells. To test this, we preincubated the wells with F3-enriched supernatants as described in Materials and Methods. Laminin, a molecule known for its coating properties, was used as a positive control. The amounts of F3 or laminin molecules adsorbed to the wells were measured by radioimmunoassay. The binding of F3 Ab was very low and approximately the same regardless of whether F3⁺ or F3⁻ supernatants had been applied, showing that the F3 molecules present did not adhere to poly-L-lysine–coated plastic substrate. The addition of FCS to the supernatant did not change the values (Table II). To definitely exclude that the effect of F3⁺ supernatant on neurite growth resulted from the action of F3 molecules immobilized on the substratum, a series of experiments was done in which DRG neurons were plated in standard medium in wells which had been preincubated for 12 h with F3⁺ supernatants (Fig. 6). After 12 or 24 h in vitro, the number of neurite-bearing cells was the same, whether the wells had been preincubated with F3⁺ (PF3⁺), F3⁻ (PF3⁻), or standard medium (S). Also, the density of the neuritic network was similar under the three conditions (not shown). We thus conclude that the effects of F3⁺ supernatants on DRG neurons are mediated by soluble F3 molecules.

Occurrence of Soluble F3 In Vivo

To investigate whether the temporal expression of F3 correlates with neurite outgrowth we analyzed brain and DRG tissues at different developmental stages for the expression of this molecule. We also tested CSF of mice of different ages for the presence of F3 to provide further evidence that F3

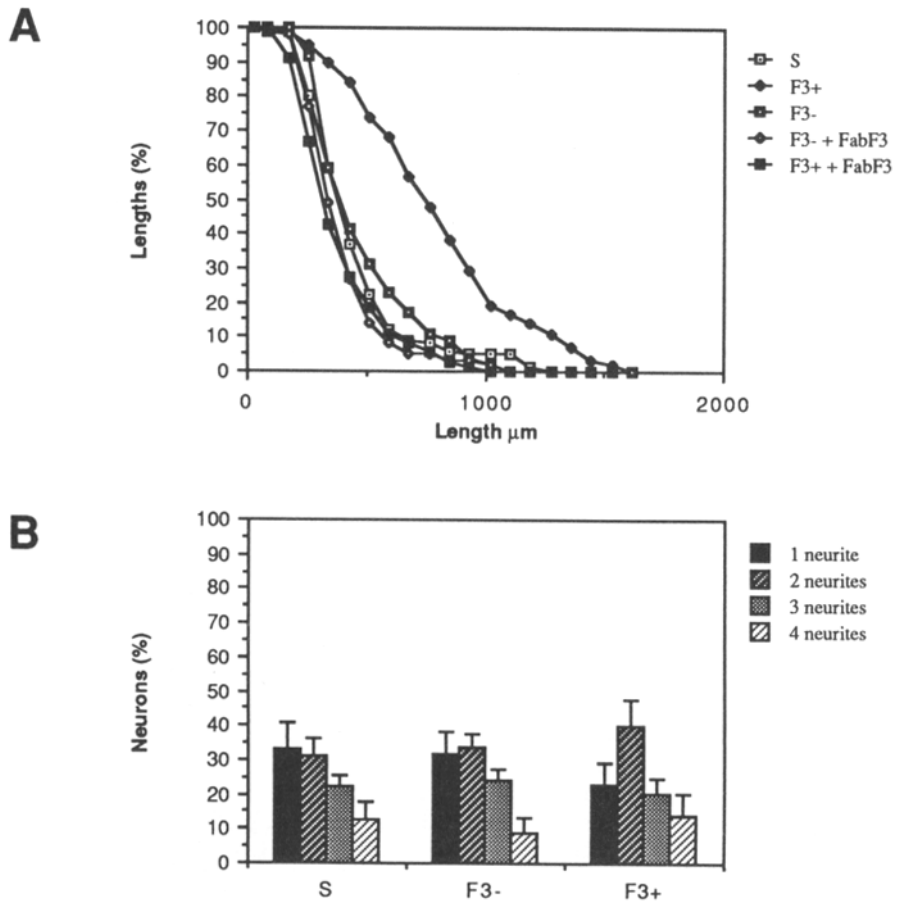

Figure 5. (*A*) Cumulative frequency distribution plot of the neurite lengths of DRG neurons after 24 h in different media in the presence or absence of anti-F3 Abs. The percentage of neurons with neurites greater than or equal to a certain length X (vertical axis) was plotted as a function of neurite length Y (horizontal axis). The values shown are from two representative experiments; mean values and the results of additional experiments are given in Table I. (*B*) Distribution of neurons according to the number of primary neurites per cell. Isolated neurons exhibiting neurite outgrowth were analyzed after 24 h and their numbers of primary neurites recorded. This is shown as the percentage of process-bearing neurons with 1, 2, 3, or 4 primary neurites in the different culture media. Culture in $F3^+$ medium resulted in a slight increase in the fraction of neurons with two or more processes at the expense of those bearing only one neurite although this increase was not statistically significant. 278, 264, and 194 neurons were counted for S, $F3^-$, and $F3^+$ medium, respectively. Bars indicate SEM values.

molecules do exist in an extracellular, soluble form in vivo. In the cerebral hemispheres, F3 became clearly detectable at postnatal day 1 (PI) with a maximum of expression between postnatal days P14 and P21, a period corresponding to intense synaptogenesis and stabilization of the neuronal network (Fig. 7 *A*). By contrast, in DRG tissues, F3 expression was very low and its expression required a long exposure of the immunoblots. Maximum occurrence was seen around embryonic day 18 followed by a sharp decrease (Fig. 7 *B*). This low expression was confirmed by immunofluores-

Table I. Analysis of Neurite Outgrowth of DRG Neurons in Different Media

Medium	Experiment No. 1	2	3	4	5	Avg. μm ± SEM
F3+	594 (40)	806 (38)	1,187 (45)	985 (35)	1,146 (36)	944 ± 245*
F3−	312 (40)	286 (48)	407 (60)	255 (76)	348 (40)	322 ± 59
S	253 (62)	270 (43)	502 (46)	304 (82)	318 (45)	329 ± 100
(F3+) + Fab F3	214 (74)	242 (58)	ND	ND	ND	227 ± 20
(F3−) + Fab F3	163 (75)	406 (39)	ND	ND	ND	284 ± 172
(F3+) + Fab NCAM	ND	ND	ND	ND	1,149 (40)	
(F3−) + Fab NCAM	ND	ND	ND	ND	323 (40)	

Neurite outgrowth was quantified by image analysis of 24-h cultures stained with Coomassie blue as described in Materials and Methods, and expressed as the total neuritic output per neuron. This was calculated as the ratio of the sum of the lengths of the neurites over the number of cell bodies. Anti-NCAM or anti-F3 Fab' fragments were added at a concentration of 200 μg/ml to the media and continuously maintained in the culture. Five independent experiments with different batches of supernatants and different DRG dissections are shown. The number of neurons analyzed per experiment is given in brackets. * indicates a significant difference ($p < 0.01$) with $F3^-$ or a standard medium according to Duncan's multiple range test (Duncan, 1955).

Table II. Analysis of the Coating Capacities of Soluble F3 and Laminin of Poly-L-lysine-treated Plastic Wells

	Antibody	−10% FCS	+10% FCS
Laminin			
1 μg/ml	+	98.6 (5.0)	100
	−	0.3 (0.4)	0.3 (0.2)
0.1 μg/ml	+	82.0 (2.5)	50.0 (10.5)
10 ng/ml	+	27.0 (0.2)	16.2 (2.1)
1 ng/ml	+	4.0 (0.6)	5.4 (0.4)
0.1 ng/ml	+	0.7 (0.2)	2.7 (0.1)
F3+	+	1.1 (0.3)	0.9 (0.1)
	−	0.3 (0.1)	0.4 (0.1)
F3−	+	0.9 (0.1)	0.9 (0.3)
	−	0.3 (0.1)	0.4 (0.1)
DME	+	0.7 (0.2)	1.6 (0.3)
	−	0.3 (0.2)	0.3 (0.1)

Serial dilutions of laminin solution, $F3^+$ or $F3^-$ supernatants, or DME alone either containing or not containing 10% FCS were incubated in plastic wells pretreated with poly-L-lysine as described in Materials and Methods. Wells were then incubated with or without antilaminin or anti-F3 Abs (dilution 1:250). After washing, the bound Abs were revealed by incubation with ^{125}I-protein A followed by γ counting. The maximum of ^{125}I-protein A bound (i.e., corresponding to wells coated with 1 μg/ml of laminin) was 16,400 cpm, and was taken as the 100% value. Other data are given in percentage. Omission of the Ab or of coating with the dilutions gave the nonspecific binding. Note that the values obtained with $F3^+$ supernatant are not significantly higher than the ones obtained with $F3^-$. This indicates that F3 molecules do not stick to the wells. The presence of FCS in the medium did not modify the values.

cence of tissue sections (not shown) showing that neuronal cell bodies were very weakly labeled without any noticeable heterogeneity of staining.

In a previous study (Gennarini et al., 1989*a*), the presence of buffer-soluble F3 has been documented for brain. Here we clearly detect F3 in CSF, further confirming that this protein exists in vivo in soluble form. A control experiment with serum (*S*) excluded the possibility that the molecules detected could arise from circulating blood (Fig. 7 *C*).

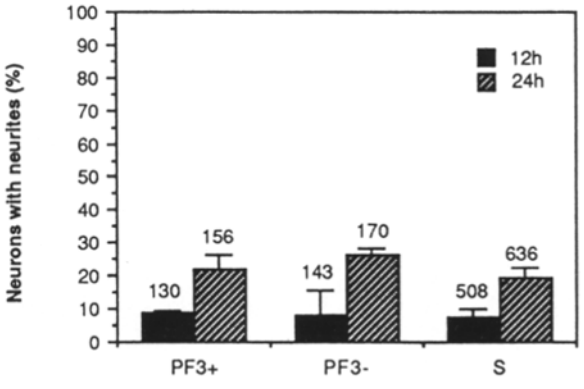

Figure 6. Analysis of the number of neurons with neurites after precoating of the wells with $F3^+$ and $F3^-$ supernatants. 0.5 ml supernatants were obtained from the cleavage of E12 ($PF3^+$) or LR-73 ($PF3^-$) cells (10^7 cells/ml) or standard medium (S) were incubated for 12 h at 37°C in poly-L-lysine plastic wells. Then, the medium was carefully drained and DRG neurons were cultured in standard medium. Data are shown for 12 and 24 h of culture, respectively. Results are the mean ± SEM for two separate experiments made with different batches of supernatants. The number of neurons counted are indicated above the bars. No statistical difference could be found between the three conditions.

Effect of CSF on Neurite Initiation and Outgrowth

Since it contains F3 (Fig. 7, *C* and *D*) in soluble form, the effect of CSF on neurite initiation and growth of DRG neurons was investigated. To this end, CSF was collected, centrifuged to remove contaminating cells and debris, and added 10 times diluted in standard medium to cultures of DRG neurons. CSF was collected from the animals used for the DRG dissection. After 13 h in culture, 32 ± 2.8% neurons (n = 793 neurons counted in six separate experiments) extended neurites in CSF-containing medium compared to 13 ± 2.6% (n = 1,060) in control medium. To determine the contribution of F3 to the observed effects, sister cultures were grown in the continuous presence of anti-F3 Fab′ fragments, in this case 20 ± 2.2% neurons (n = 492) extended neurites. Hence, F3 contributes to neurite outgrowth stimulation by CSF, but is not the only molecule responsible for the overall effect. There was no effect of anti-NCAM Abs on neurite initiation, as 14 ± 1.8% (n = 374) and 29 ± 2% (n = 402) of the neurons extended neurites in control and CSF-containing medium, respectively.

Data shown in Table III, corresponding to six independent experiments, indicated that the average neuritic length per neuron in cultures made in the presence of CSF (S + CSF) was always higher than the one measured in standard medium (S). Statistical analysis of the pooled data gave an average of 541 ± 78 μm compared with 245 ± 38 μm. As for neurite initiation, the presence of anti-F3 Fab′ fragment (S + CSF + Fab F3) was shown to partially inhibit the neurite growth-promoting effect of the CSF (541 ± 78 μm compared with 382 ± 59 μm). The effect of Fab′ anti-F3 antibody in reducing neurite outgrowth seems specific, as the measured values were not affected by the presence of equivalent concentrations of Fab′ anti-NCAM antibody. These observations further confirm that F3 present in CSF plays a role similar to the one described for F3 in the PI-PLC supernatants and show that it is active in soluble form.

Discussion

F3 is an immunoglobulin superfamily protein found on subsets of central and peripheral neurons that is anchored to the plasma membrane by GPI and is prominently expressed on axons and neuropil (Gennarini et al., 1990; Faivre-Sarrailh et al., 1992). Our previous results (Gennarini et al., 1991) have established two points. First, F3 fulfills the operational definition of a cell-cell adhesion molecule. Second, the molecule stimulates neurite outgrowth when presented to sensory neurons as a surface component of transfected CHO cells on which the neurons are grown. A number of cell adhesion molecules are known to enhance the neurite outgrowth when offered as a substratum to cultured neurons either by adsorbing the purified molecules to the culture dish (Lagenaur and Lemmon, 1987; Chang et al., 1987; Furley et al., 1990) or, as in this case, by expressing them on a monolayer of cells used as a substrate (Johnson et al., 1989; Filbin et al., 1990; Doherty et al., 1990; Schneider-Schaulies et al., 1990). In principle, there are two ways in which cell adhesion molecules could accomplish this. They may act mechanically by providing a more adhesive substrate favorable for neurite extension. Alternatively, their binding to a neuronal receptor may trigger intracellular signals that are responsible for neurite outgrowth stimulation. The latter possibility is sup-

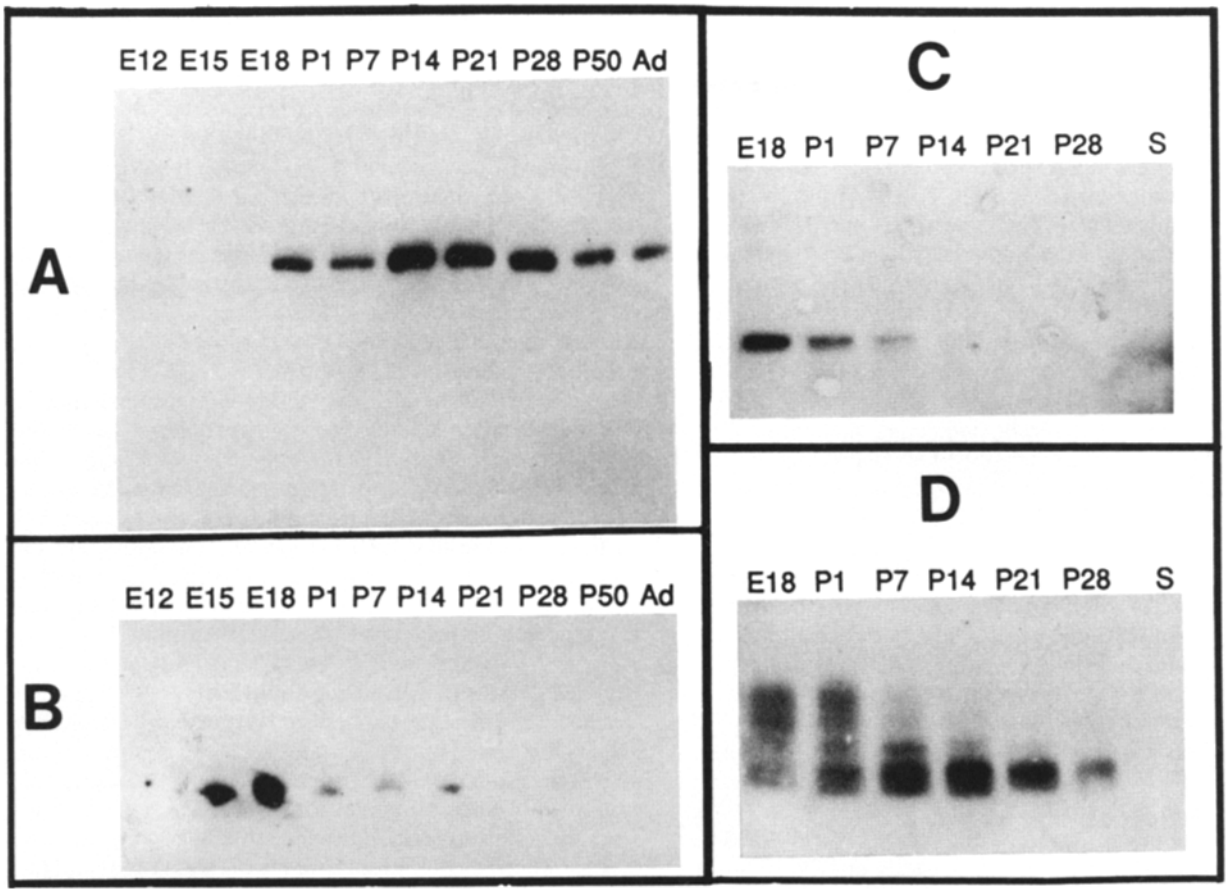

Figure 7. Immunoblot analysis of F3 expression during nervous system development and in CSF. Homogenates of cerebral hemispheres (*A*) or DRGs (*B*) (30 μg protein) prepared as described in Materials and Methods or 10 μl of CSF (*C* and *D*) were analyzed by immunoblotting using anti-F3 (1:250) (*A–C*) polyclonal Abs. Anti-NCAM Abs (1:1,000) were used to reveal this molecule in the CSF (*D*). The level of F3 molecules expressed by DRG tissues (*B*) was very low as its detection necessitated 72 h of autoradiographic exposure by comparison to 10 h for detection in brain (*A*) or CSF (*C*). *E*, embryonic day; *P*, postnatal day.

ported by studies on PC12 cells showing that triggering of adhesion molecules at the surface by Ab binding results in changes in second messenger system (Schuch et al., 1989) and that Ca^{2+} channel blockers and pertussis toxin inhibit neurite outgrowth in response to NCAM and *N*-cadherin (Doherty et al., 1991). To address this question, we have devised a simple and effective scheme to prepare F3 in soluble form after release from the cell surface. We show that this

Table III. Analysis of Neurite Outgrowth in CSF-containing Cultures

	Experiment No.						
Medium	1	2	3	4	5	6	Avg. μm ± SEM
S + CSF	410	529	529	547	645	586	541 ± 78
	(51)	(42)	(51)	(40)	(40)	(41)	
S	171	250	281	268	244	256	245 ± 38
	(51)	(55)	(53)	(42)	(42)	(41)	
S + CSF + Fab F3	320	437	390	ND	ND	ND	382 ± 59
	(50)	(48)	(43)				
S + Fab NCAM	ND	ND	ND	276	267	249	264 ± 14
				(40)	(46)	(44)	
S + CSF + Fab NCAM	ND	ND	ND	528	616	617	587 ± 51
				(42)	(40)	(40)	

Neurite outgrowth was quantified by image analysis of 13-h cultures stained with Coomassie blue and expressed as the neuritic output/neuron in μm. CSF was collected from the postnatal mice used for the DRG dissection and added 1:10 diluted to standard medium (S + CSF). Sister cultures were grown in the continuous presence of anti-F3 or anti-NCAM Fab' fragments (200 μg/ml) (S + CSF + Fab F3, S + CSF + Fab NCAM, respectively). Standard medium was used as a control (S). Control for nonspecific Fab effect on growth were performed by adding anti-NCAM Fab' fragments to standard medium (S + Fab NCAM) and to cultures made in presence of CSF (S + CSF + Fab NCAM). Six independent experiments are shown. The number of neurons analyzed per experiment is given in brackets.

material is very effective in stimulating neurite initiation and neurite extension and provide evidence that the soluble F3 molecules are responsible for these effects. As it is difficult to envisage how soluble F3 molecules may exert their effects by purely mechanical means, the most plausible interpretation of our results is that F3 acts as a ligand capable of inducing transmembrane signaling.

Our conclusion that soluble F3 promotes initiation and extension of neurites is based on the following. First, the initial outgrowth of neurites and the density of the neuritic network produced by DRG neurons were stimulated in F3-containing medium as compared with similarly prepared medium that lacked the molecule. By contrast, there was no difference between standard medium and the one recovered after PI-PLC treatment of the control cells indicating that other GPI-anchored proteins that are expressed on CHO cells, including NCAM-120, have no effect. Second, addition of monovalent anti-F3 Abs, but not of anti-NCAM Abs used as control, reduced the number of process-bearing neurons and the neuritic output per neuron to control levels. Third, the intensity of the response to F3-containing medium was dose-dependent while dilution of the control supernatant had no effect on neurite outgrowth thus excluding an aspecific medium effect. Although we are unable to give a precise value of the F3 concentration present, a comparison with NCAM-120 levels suggests that it should be in the nanomolar range. Fourth, the F3 molecules in the $F3^+$ medium do not seem to be active in a form adsorbed to the substratum, since precoating the culture dishes with soluble F3 had no effect on the measured parameters. Finally, addition of CSF, a natural source of soluble F3, to standard culture medium also stimulated neurite extension, and this effect was partially blocked in the presence of anti-F3 antibodies.

There are of course ways other than transmembrane signaling, in which soluble F3 could bring about the observed effects, that deserve consideration. The most obvious one is that soluble F3 acts as a competitive inhibitor of adhesion mediated by the membrane-bound form. An antiadhesive function has been postulated for released forms of cell–cell adhesion molecules, such as cell-CAM 120/80 (Wheelcock et al., 1987) and NCAM-120 (Hé et al., 1987). Relatively high concentrations of soluble axonin-1, a neurite outgrowth-promoting molecule, have been found to inhibit neurite fasciculation (Stoeckli et al., 1991) providing experimental evidence for such an antiadhesive effect. However, our data is difficult to reconcile with this or a similar mechanism. In our hands, DRG-neurons that did not bear detectable levels of F3 at their surface as determined by immunostaining (data not shown), and that were cultured on an artificial poly-L-lysine substrate, responded with increased neurite outgrowth making it highly unlikely that soluble F3 functions as an inhibitor of F3-mediated adhesion. In any case, we observed enhancement of neurite outgrowth on isolated neurons which excludes an effect on cell–cell adhesion while interference with cell–substrate adhesion, which might favor growth cone navigation, is improbable on a poly-L-lysine substrate, unless one postulates on interaction with a component deposited onto the poly-L-lysine by the cells.

Soluble F3 molecules may also in some way interfere with a negative signaling event mediated by another ligand that inhibits neurite growth. Even if this explanation turned out to be correct, an unavoidable conclusion would be that F3 participates in a regulatory circuit and cannot function merely by modulating directly cell–cell or cell–substrate adhesion. $F3^+$ medium does not seem to act by favoring the survival of a subclass of DRG neurons since cell attachment survival approached 80% and was not modified by the presence of $F3^+$ medium. Examination of cell morphology parameters such as the size of the cell bodies and the number of primary neurites did not yield any indication for the selection of a distinct subpopulation. In fact, the continuous shift of neurite lengths towards higher values in cumulative frequency plots suggests that all neurons which had initiated neurites responded to $F3^+$ medium. The receptor for F3 seems thus widely distributed on sensory neurons.

Our results raise several important questions. One is whether the same type of mechanisms mediate the neurite outgrowth-promoting properties of both membrane-bound and soluble F3. An extreme view would be that our previous results, showing that DRG neurons extend longer processes on F3-expressing than on F3-negative monolayers, are entirely due to soluble F3 released by the cells. Such a mechanism is possible since transfected CHO cells release substantial amounts of F3 protein into the culture medium (Gennarini et al., 1991). The effects of soluble F3 and F3-bearing monolayer cells on the density of the neuritic network were similar, the main difference being that neuritic outgrowth promotion by the monolayer cells was delayed as compared with the effect of soluble F3. This could be taken to mean that F3 molecules released into the medium were the active component, since it would take some time to build up the required concentration of soluble molecules in the medium. It is plain, however, that additional experiments are needed to prove the point, and we cannot exclude the possibility that membrane-bound and soluble F3 act by different mechanisms.

A second question concerns the nature of the receptor at the neuronal cell surface with which F3 interacts and the identification of its molecular nature thus becomes a major goal of future experimentation. The ability of F3 transfectants to form mixed aggregates with parental cells (Gennarini et al., 1991) indicated that they bind to a receptor constitutively expressed by the CHO cells, implying that F3-mediated adhesion is heterophilic. Results from another study further corroborate this idea. In a recent ultrastructural study of F3 localization in the developing cerebellum (Faivre-Sarrailh et al., 1992), F3 molecules were found to be prominently expressed at three types of synaptic sites. In each type of synapse, F3 was found either pre- or postsynaptically but never at both sites, suggesting that it is engaged in heterophilic interactions where neurons are in contact.

It has been shown previously (Gennarini et al., 1990) that F3 exists in brain tissue in an apparently soluble form. This is substantiated further by our present results showing that F3 is present in the CSF during the perinatal period in concentrations sufficient to produce a detectable neurite outgrowth-promoting effect. It is thus reasonable to assume that soluble F3 is present also in the interstitial space of the brain in high enough concentrations and at the right times to affect axonal growth. Occurrence in both surface-associated and released forms seems to be widespread among neuronally expressed proteins with adhesive function. Known examples include the closely related protein TAG-1 (Furley et al., 1990; Karagogeos et al., 1991), other immunoglobulin superfamily members such as NILE/L1 (Sweadner, 1983), and

the GPI-linked isoform of NCAM (Gennarini et al., 1986; Bock, 1987), as well as axonin-1 (Ruegg et al., 1989), a protein of still unknown primary structure. The physiological relevance of this phenomenon has remained enigmatic and various functions have been proposed for the release process itself and for the soluble molecules. Only in the case of axonin-1 has it been shown that the soluble form is indeed functional, but the observed antiadhesive effect occurred only at unphysiologically high concentrations (Stoeckli et al., 1991). Our results strongly suggest that the soluble forms of adhesive proteins with neurite outgrowth-promoting properties could induce enhanced growth of neuronal processes not by modulating adhesion but by binding to neuronal cell surface receptors. In this way they may be able to act at some distance from their site of release much in the same way as local growth and trophic factors do. Further experiments should indicate whether the growth-promoting effect attributable to soluble F3 we demonstrated in vitro is also operative in normal development. This would put F3 soluble molecules among a class of factors which, by encouraging neuritic growth, could be involved in sculpting neuronal morphology.

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