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 glycans-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.

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1. Abbreviations used in this paper: Ab, antibody; CSE cerebrospinal fluid; DRG, dorsal root ganglia; GPI, glycosylphosphatidylinositol; NCAM, neuronal 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 transfectedants, the cDNA codes for a 142-kD GPI-anchored protein. F3-expressing transfectedants, 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 transfectedants we used in our assay. This observation raises questions as to the possible form of soluble F3, cells were plated in F3 + or F3- supernatants obtained as described below. In some experiments, dilutions of F3 + or F3- supematants containing 2% (wt/vol) Fab' fragments of anti-F3 or neural cell adhesion molecule (NCAM) Abs were 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., 1996b). 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., 1989c). A site-directed rabbit polyclonal anti-NCAM Ab recognizing the NH2-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 antiseraum raised against immunopurified adult mouse NCAM (Gennarini et al., 1986).

Preparation of F3-enriched Supernatant

Parental or transfected CHO cells were harvested in Gibeo-BRL (Cergy-Pontoise, France). For experiments designed to test the effects of soluble F3, cells were plated in F3+ or F3- supernatants as described below. In some experiments, dilutions of F3+ or F3- supernatants or 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.
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., 1989b). Later on, F3 in mouse brain homogenates was found to be distributed between a buffer-soluble and a membrane fraction (Gennarini et al., 1989a). 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-cm² 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.
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.

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 El2 suggesting that the values recorded with undiluted El2 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 cm\(^2\) 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 length
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. 5A, 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 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-cm² 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.
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-precess-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., 1989a), 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. 5A) confirm that anti-F3 Abs completely abolish the effect of F3+ medium. 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 neurite 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 I). 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 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
molecules do exist in an extracellular, soluble form in vivo. In the cerebral hemispheres, F3 became clearly detectable at postnatal day 1 (P1) with a maximum of expression between postnatal days P14 and P21, a period corresponding to intense synaptogenesis and stabilization of the neuronal network (Fig. 7A). 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. 7B). This low expression was confirmed by immunofluores-

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

| Medium | Experiment No. | Avg. Length (μm) ± SEM |
|--------|----------------|------------------------|
| F3+    | 1 | 2 | 3 | 4 | 5 | 944 ± 245* |
|        | (594) | (806) | (1,187) | (985) | (1,146) |
| F3-    |        | | | | |
|        | (312) | (286) | (407) | (255) | (348) |
| S      | (253) | (270) | (502) | (304) | (318) |
| (F3+) + Fab F3 | (214) | (242) | ND | ND | 227 ± 20 |
| (F3-) + Fab F3 | (163) | (406) | ND | ND | 284 ± 172 |
| (F3+) + Fab NCAM | ND | ND | ND | 1,149 | (40) |
| (F3-) + Fab NCAM | 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).
Heterogeneity of staining.

The presence of tissue sections (not shown) showing that neuronal and Laminin of Poly-L-lysine-treated Plastic Wells could arise from circulating blood (Fig. 7 C).

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 were with poly-L-lysine as described in Materials and Methods.

The numbers in Table I, 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/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; Furlay 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-Schaule 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-

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

| Laminin | Antibody | -10% FCS | +10% FCS |
|---------|----------|----------|----------|
| 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.

The number of neurons with neurites after precoating of the wells with F3+ or F3− supernatants. 0.5 ml supernatants were obtained from the cleavage of E12 (PF3−) or LR-73 (PF3+) cells (10⁵ 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 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.

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**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 (10⁵ 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.
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.

reported 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++ 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. | 1 | 2 | 3 | 4 | 5 | 6 | Avg. μm ± SEM |
|---------------|---|---|---|---|---|---|--------------|
| Medium        |   |   |   |   |   |   |              |
| S + CSF       | 410 | 529 | 529 | 547 | 645 | 586 | 541 ± 78     |
| S             | 171 | 250 | 281 | 268 | 244 | 256 | 245 ± 38     |
| S + CSF + Fab F3 | 320 | 437 | 390 | ND | ND | ND | 382 ± 59     |
| S + Fab NCAM  | ND | ND | ND | 276 | 267 | 249 | 264 ± 14     |
| S + CSF + Fab NCAM | ND | ND | ND | 528 | 616 | 617 | 587 ± 51     |

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-1-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-1-lysine substrate, unless one postulates on interaction with a component deposited onto the poly-1-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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References

Bock, E., K. Edvardsen, A. Gibson, D. Linnenmann, J. Lyles, and O. Nybroe. 1987. Characterization of soluble forms of NCAM. FEBS Lett. 225:33-36.

Brumendorf, T., J. M. Wolff, R. Frank, and F. G. Rathjen. 1989. Neural cell recognition molecule F11: homology with fibronectin type II and immunoglobulin type C domains. Neuron. 2:1351-1361.

Chang, S., F. G. Rathjen, and J. A. Raper. 1987. Extension of neurites on axons is impaired by antibodies against specific neural cell surface glycoproteins. J. Cell Biol. 105:255-262.

Dodd, J., and T. Jessell. 1988. Axonal guidance and the patterning of neuronal axons is impaired by antibodies against specific neural cell surface glycoproteins. J. Cell Biol. 109:1239-1244.

Rathjen, F. G., and M. Schachner. 1984. Immunocchemical and biochemical characterization of a new neuronal cell surface component (LI) which is involved in cell adhesion. EMBO J. 3:3-10.

Ratjen, F. G., J. M. Wolff, S. Chang, F. Bonhoeffer, and J. A. Raper. 1987a. Neurofascin: a novel chick cell surface glycoprotein involved in neurite-neurite interactions. Cell. 51:841-849.

Doherty, P., M. Fruns, P. Seaton, G. Dickson, C. H. Barton, T. A. Sears, and F. S. Walsh. 1990. A threshold effect of the major isoforms of NCAM on neurite outgrowth. Nature (Lond.). 343:464-466.

Doherty, P., S. Ashton, S. Moore, and F. Walsh. 1991. Morphoregulatory activities of NCAM and N-Cadherin can be accounted for by G protein-dependent orientation of L- and N-type neuronal Ca2+ channels. Cell. 67:21-33.

Dunlop, F. W., and A. Griewe. 1975. A simple technique for eliminating interference by detergent in the Lowry method of protein determination. J. Biolum. Chem. 261:3293-3291.

Gennarini, G., G. Cibelli, G. Rougon, M. G. Mattei, and C. Goridis. 1989a. The mouse neuronal cell surface protein F3: a phosphatidylinositol-anchored member of the immunoglobulin superfamily related to chicken contactin. J. Cell Biol. 109:776-786.

Gennarini, G., G. Rougon, F. Vitiello, P. Corsi, C. Di Benedetta, and C. Goridis. 1989b. Identification and cDNA cloning of a new member of the L2/HNK-1 family of neural surface glycoproteins. J. Neurosci. Res. 22:31-42.

Gennarini, G., G. Rougon, and C. Goridis. 1990. F3: a new developmentally regulated member of the HNK-1 family. Acta. Histochem. Band XXXVIII (Suppl.): S65-S69.

Gennarini, G., P. Durbec, A. Boned, G. Rougon, and C. Goridis. 1991. Transfected F3/F11 neuronal cell surface protein mediates intercellular adhesion and promotes neurite outgrowth. Neuron. 6:595-606.

Hé, H. T., J. Finne, and C. Goridis. 1987. Biosynthesis, membrane association, and release of NCAM-200, a phosphatidylinositol-linked form of the neural cell adhesion molecule. J. Cell Biol. 105:2489-2500.

Johnson, P. W., W. Abramow-Newley, B. Seilheimer, R. M. Tropak, M. Arquint, R. J. Dunn, M. Schachner, and J. C. Roder. 1989. Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. Neuron. 3:377-385.

Karagegoz, D., S. M. Fenton, F. Casano, J. Dodd, and T. M. Jessel. 1991. Developmental expression of the axonal glycoprotein TAG1: differential regulation by central and peripheral neurons in vitro. Development (Camb.). 112:51-67.

Lagrene, C., and V. Lemmon. 1987. An L1-like molecule, the 8D9 antigen, is a potent substrate for neurite extension. Proc. Natl. Acad. Sci. USA. 84:775-777.

Letourneau, P. C. 1987. What happens when growth cones meet neurites: attraction or repulsion. Trends Neurosci. 10:390-393.

Low, M. G., and A. R. Saliet. 1988. Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science (Wash. DC). 239:270-275.

Moos, M., R. Tacke, H. Sherrer, D. Teplov, K. Frith, and M. Schachner. 1988. Neural adhesion molecule L1 as a member of the immunoglobulin superfamily with binding domains similar to fibronectin. Nature (Lond.). 334:701-703.

Pollard, J. W., and C. Stanners. 1979. Characterization of cell-surface glycoconjugates involved in growth control isolated from both the wild-type and a lecHtRNA synthetase mutant of Chinese hamster ovary cells. J. Cell Physiol. 98:571-581.

Sweadnez, K. 1983. Size, shape and solubility of a class of releasable surface proteins of sympathetic neurons. J. Cell Biol. 97:21-33.

Rathjen, F. G., and M. Schachner. 1984. Immunocchemical and biochemical characterization of a new neuronal cell surface component (L1) which is involved in cell adhesion. EMBO J. (Eur. Mol. Biol. Organ.) 3:3-10.

Roege, M. A., E. T. Stoeckli, R. B. Lanz, P. Strelt, and P. Sonderberger. 1989. A homologue of the axonally secreted protein axonin-1 is an integral membrane protein of nerve fiber tracts involved in neurite fasciation. J. Cell Biol. 109:2363-2378.

Rsualati, E. 1988. Fibronectin and its receptors. Annu. Rev. Biochem. 57:375-413.

Schneider-Isakiewicz, J., A. Von Bronn, and M. Schachner. 1990. Recombinant peripheral myelin protein P0 mediates both adhesion and neurite outgrowth-promoting properties. J. Neurosci. 7:286-297.

Schuch, U., M. Lohse, and M. Schachner. 1989. Neural cell adhesion molecules influence second messenger systems. Neuron. 3:13-20.

Stoeckli, E., T. B. Kuhn, C. O. Duc, M. A. Rung, and P. Sonderberger. 1989. The axonal secreted protein axonin-1 is a potent substratum for neurite growth. J. Cell Biol. 112:449-455.

Swans, K. 1983. Size, shape and solubility of a class of releasable surface proteins of sympathetic neurons. J. Cell Biol. 98:2512-2519.

Takeichi, M. 1988. The cadherin: cell-cell adhesion molecules controlling animal morphogenesis. Development (Camb.). 102:639-655.

Thieves, S., P. Malapert, and G. Rougon. 1990. Antibody against B. burgdorferi lipoprotein 28-kDa protein blocks B. burgdorferi lipoprotein 28-kDa protein blocks. Immunol. Cell Biol. 68:87-93.

Wheelock, C., B. Bechtol, and C. Damasky. 1987. Soluble 80 kDa fragment of cell-CAM 120/80 disrupts cell-cell adhesions. J. Cell Biol. 109:187-208.

Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfAMILY: domains for cell surface recognition. Annu. Rev. Immunol. 6:381-405.

Durbec et al. Role of Soluble F3 Molecules

887