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ARTICLES

Expression and Release of Phosphatidylinositol Anchored Cell Surface Molecules by a Cell Line Derived From Sensory Neurons

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Abstract Early postnatal mouse dorsal root ganglion neurons were found to express several glycosylphosphatidylinositol-anchored (GPI) molecules from the immunoglobulin superfamily (neural cell adhesion molecule 120 kD isoform, F3, Thy1) whose expression is developmentally regulated. A hybrid cell line (ND26), made by fusing postmitotic rat dorsal root ganglion (DRG) neurons with the mouse neuroblastoma N18Tg2, could be induced to differentiate by manipulating the composition of the culture medium and expressed similar GPI molecules to DRG neurons. We used this model system to investigate the metabolism of GPI-anchored molecules. We found that neural cell adhesion molecule 120 Kd isoform expression decreased upon differentiation, whereas the level of F3 and Thy1 increased, suggesting a role in neurite outgrowth processes.

The ratio of molecules cleavable by exogenous phosphatidylinositol phospholipase C (PI-PLC) was similar for all the GPI-anchored molecules, which could mean that cell-specific modifications of the basic anchoring structure determine the level of potentially releasable molecules. Measurements of spontaneous release indicated that this reflected the overall level of expression of these molecules by the ND26 cell line.

Finally, we observed an effect of dibutyryl cAMP on the level of expression of F3 and Thy1 but not of N-CAM. However, we could not detect any significant effect of nerve growth factor (NGF) either on the level of expression or on the amount of spontaneously released molecules.

Key words: differentiation, neuronal cells, GPI-anchored molecules, metabolism

The growth of neuritic processes is one of the first cellular events leading to the differentiation of the neuronal cell. This event involves many interactions between axons, neighbouring cells, and the extracellular matrix [Dodd and Jessell, 1988]. Potential mechanisms by which axons adapt to a changing cellular environment involve spatio-temporal regulation of glycoprotein expression, either on individual cells or on different segments of the same axon, post-translational modifications of pre-existing molecules, and modification of the extracellular matrix by deposition of new molecules.

Many glycoproteins have been identified and shown to play a role in neurite extension and fasciculation [Doherty and Walsh, 1989; Jessell, 1988]. Several of them are adhesion molecules which, on the basis of their predicted primary structure, have been grouped into larger families. Most of them belong to the immunoglobulin superfamily [Williams and Barclay, 1988]. The most studied of these are neural cell adhesion molecules (N-CAM) [Edelman, 1988] and L1 [Ratjen and Schachner, 1984]. Thy1, the smallest member of this family, is also expressed on

Abbreviations used: Bt2cAMP dibutyryl cAMP; DMEM, Dulbecco's modified Eagle medium; DRG, dorsal root ganglia; FCS, fetal calf serum; GPl, glycosylphosphatidylinositol; MAP, microtubule-associated protein; Mr., molecular weight; N-CAM, neural-cell adhesion molecules; NGF, nerve growth factor; PBS, phosphate buffered saline; PI-PLC, 1-phosphatidyl-D-myoinositol phosphohydrolase EC3.1-4-10 (cyclic phosphate forming); PMSF, phenylmethylsulfonylfluoride.

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neural cells although its role in neurite outgrowth has not been clearly demonstrated [Liesi et al., 1990].

The recent sequencing of the neuronal surface protein F3 in mouse [Gennarini et al., 1989a,b] had led to the definition of a new member of the immunoglobulin superfamily. This protein is prominently expressed on neuronal processes rather than on the corresponding cell bodies in long-term primary cultures [Gennarini, 1989b]. It is anchored to the plasma membrane by a GPI-group. This post-translational modification [Ferguson and Williams, 1988] is expressed by Thy1 [Tse et al., 1985] the smallest member of the immunoglobulin superfamily also found on neural cells, and by the smallest isoform of N-CAM, N-CAM120 [Hé et al., 1986].

One feature of the GPI-anchored molecules is that they are found as either membrane-bound or soluble forms spontaneously released by cells [Hé et al., 1987]. The diversity of GPI-anchored molecules expressed by developing neurons and their existence as membrane-bound or soluble forms suggests that they could play a role in neural development. The mechanisms implicated in their liberation have not been elucidated. So far, it is not known whether release is controlled by mechanisms specific to groups of molecules involved in similar functions or dependent of the GPI-anchored. In the last case, release could be specific to each GPI-anchored molecule or common to all molecules expressed by a given cell. Observation of GPI-anchored molecule expression and monitoring of release during neuronal differentiation should give some insight into the functions of these molecules.

In this study, we first assessed on primary cultures of DRG neurons the expression of three GPI-anchored molecules from the immunoglobulin superfamily among which at least two (N-CAM and F3) are thought to play a role in neurite outgrowth and fasciculation. While such cultures allow spatio-temporal expression of molecules to be analysed by immunofluorescence staining, biochemical studies are often frustrated by difficulties in obtaining an adequate number of cells. To overcome these difficulties, we used an immortalised cell line, termed ND26, obtained by fusing post-mitotic rat DRG neurons with a mouse neuroblastoma cell line (N18Tg2) [Wood et al., 1990]. We showed that this cell line faithfully expressed properties of its sensory neuron parent and that its state of differentiation could be controlled by manipulating the composition of the culture medium. This model was then used to monitor the expression and the release of GPI-anchored molecules under various conditions.

MATERIALS AND METHODS

Materials

Tissue culture media were supplied by Gibco BRL (France). Plastic for tissue culture was supplied by Nunc (France). [^{125}I]Na (carrier free) was purchased from Amersham (GB). Protein A–Sepharose coated beads were from Pharmacia (France). Sulfo-NHS-biotin was from Pierce (USA). All other reagents were from Sigma (USA). Pl-PLC enzyme was purified in our laboratory from *B. thuringiensis* [Théveniau et al., 1990]. Iodine-[^{125}I] labelled protein A and streptavidin were iodinated in our laboratory with a specific radioactivity of 1 Ci/mmol.

Cell Culture

The ND26 cell line originated from a panel of hybridomas made by fusing rat DRG neurons with a mouse neuroblastoma cell line N18Tg2 [Wood et al., 1990]. In standard condition, these cells were grown in 50% Dulbecco's modified Eagle medium (DMEM)/50% HamsF12 supplemented with 10% fetal calf serum (FCS) and 50 IU/ml penicillin, 50 μg/ml streptomycin. For our experiments, the cells were maintained in defined medium composed as follows: 50% DMEM, 50% HAMsF12 buffered with Hepes (pH 7.4), supplemented with 1.2 g/l $NaHCO_3$, 50 μg/ml human transferrin, 30 nM selenium, 100 μM putrescin, 20 nM progesterone, 1 pM β estradiol, and antibiotics. This medium will be referred to as *minimum medium*. When mentioned, 1 mM dibutyryl cAMP (Bt2cAMP) and/or 50 ng/ml NGF were also added.

Dorsal Root Ganglia Cultures

Primary cultures were prepared on poly-L-lysine coated glass coverslips or Petri dishes from DRG [Fields et al., 1978] of 1-day-old mice, and grown in 10% FCS medium supplemented with 50 ng/ml NGF for 1 or 3 days before experiments. For some experiments this medium was changed for minimum medium supplemented with NGF.

Antibodies

Different antibodies were used: a site directed rabbit antisera anti-N-CAM recognizing the NH_2-

terminal domain of all the isoforms [Rougon and Marshak, 1986], and a rat monoclonal antibody (H28) recognizing mouse N-CAM [Hirn et al., 1981]. A polyclonal anti-Thy1 antibody (prepared in our laboratory), an anti-F3 rabbit polyclonal [Gennarini et al., 1989b]. An anti-MAP1 (microtubule associated protein 1) mouse monoclonal antibody (a kind gift from Dr. C. Garner, Hamburg, FRG) [Tucker et al., 1988]. Affinity purified goat anti-rabbit, anti-rat, and anti-mouse $F(ab)_2$ fragments labelled either with fluorescein or with rhodamine were from Immunotech (France).

Immunofluorescence Staining

Cells were seeded on poly-L-Lysine coated coverslips and maintained in minimum medium containing 50 ng/ml NGF. Immunofluorescence staining was realized after various periods of time as described by Rougon et al. [1983]. For visualization of cytoskeletal proteins, anti-MAP antibodies were used after permeabilization of the cells for 5 min in a 50% acetic acid/50% ethanol solution at −20°C. In all cases the primary and secondary antibodies were incubated for 45 min at room temperature. Coverslips were examined under a Olympus microscope and photographs taken on ASA 400 TRI-X Kodak films.

Immunoprecipitation

Cells were kept for 3 days in defined medium. Then sulfo-NHS-biotin labelling was performed with 0.5 mg/ml sulfo-NHS-biotin in PBS containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, for 20 min at room temperature according to Lisanti et al. [1988]. Cells were washed, scraped, and recovered in the same buffer supplemented with 0.5 mM phenylmethylsufonyl fluoride (PMSF) and 5 μg/ml α2-macroglobulin as protease inhibitors. After centrifugation, cells were resuspended in the same buffer as above and incubated for 1 h at 37°C with or without 0.01 IU/ml of PI-PLC. Supernatants were recovered by ultracentrifugation at 100,000*g* for 30 min in a TL100 Beckman ultracentrifuge. Pellets were solubilized in RIPA buffer: 50 mM Tris-HCl (pH 7.4) buffer containing 150 mM NaCl, 1% Nonidet P40, 1% deoxycholate, 0.1% sodium dodecylsulfate, and protease inhibitors. Then the suspension was submitted to one cycle of freezing and thawing, and centrifuged for 30 min at 100,000*g*.

Supernatants and RIPA solubilisates of the pellets were used for immunoprecipitation. Preclearing was done with 50 μl Protein A–Sepharose coated beads for 15 h at 4°C. Then immunoprecipitation was carried out for 5 h at 4°C with preformed complexes made by preincubation of antibodies (5 μl of serum) with 20 μl of Protein-A beads. Immunoprecipitates were submitted to three washes in three different buffers as described [Gennarini et al., 1984] and boiled in reducing electrophoresis sample buffer. Samples were loaded on 7 or 12% SDS-polyacrylamide gels as a function of the molecular weight (Mr.) of analysed molecules, which were detected after blotting onto nitrocellulose™ membrane (Amersham) by incubation with 10^6 cpm/ml streptavidin and subsequent autoradiography.

Protein Slot Analysis

Cells (15×10^3 cells/well) were seeded in 96-well plates coated with poly-L-lysine. They were maintained in minimum medium containing when needed either 1 mM Bt2cAMP and/or 50 ng/ml NGF for 4 days. Medium was changed every day in order to keep up a steady level of factors, and recovered. Media from the two days and from the two following days of culture were pooled and slot-blotted onto nitrocellulose sheets.

GPI molecules expressed at the cell surface were analysed by treatment with PI-PLC (0.01 IU/ml) for 1 h at 37°C, and slot-blotting of the recovered incubation media. This experiment was performed after 2 and 4 days in culture with defined medium.

Similarly, the presence of non–PI-PLC cleaved molecules, either resistant or intracellular, was investigated by slot-blotting of cells solubilized in 20 mM TRIS-HCl buffer (pH 8.5) containing 1% deoxycholate, 20 mM NaCl, 1 mM $MgCl_2$, 5 mM dithiothreitol, 0.5 mM PMSF, and 5 μg/ml α2 macroglobulin.

After slot-blotting of proteins, nitrocellulose sheets were washed in phosphate buffered saline (PBS) and saturated for 1 h in PBS–5% defatted milk. Incubation with the relevant antibodies was conducted for 16 h at 4°C and bound antibodies were detected with 0.5×10^6 cpm/ml [^{125}I] protein A. Sheets were autoradiographed using Fuji X-Ray films. Care was taken to use saturating levels of antibodies and the same batch of [^{125}I] protein A in each series of experiments, in order to be able to perform quantitative estimations. Experiments were conducted

at least in triplicate and on 4 wells for each given condition.

Quantification of the Data

Immunoslot densitometric analysis was conducted by image analysis of autoradiogram exposures performed in the linear range of sensitivity of the film, using a digitized program Biolab TM with Macintosh Programming Workshop for Macintosh II. In this program images are digitized to 8 bits (256 grey levels) with picture definition of 768 × 576 pixels (picture elements). Image acquisition is handled by camera CCD. Grey levels were measured by using a superimposed rectangular window; the values obtained were stored and transferred to the statistical program. In some instances radioactive slots were cut from the nitrocellulose sheet and individually counted in a gamma-counter.

RESULTS

Expression of GPI-Anchored Molecules by Developing DRG Neurons

Primary cultures prepared from 1 day postnatal mouse and cultured in 10% FCS medium containing NGF were chosen for the analysis of the expression of GPI-anchored glycoproteins. Visualization of these molecules was conducted first by immunofluorescence staining. After one day in culture all neurons strongly expressed N-CAM on their surface whereas F3 and Thy1 were hardly detectable. After 3 days in vitro, cells grew dense neuritic networks and expressed Thy1 and F3 on their cell bodies and on their neurites (Fig. 1B–D). Not every neuron expressed F3 and the expression of this molecule was restricted to large neurons as already reported [Gennarini et al., 1989b].

The spontaneous release of these molecules from the membrane of DRG neurons was assessed by probing the culture medium with relevant antibodies. To this end, the cell surface of cultures maintained for 3 days in vitro was biotinylated as described in Methods; then the cells were incubated for 1 h in biotinylation buffer. Soluble fractions were collected, cells were lysed in RIPA buffer, and samples were submitted to sequential immunoprecipitation with the antibodies. Then the immunoprecipitated molecules were run on SDS-PAGE under reducing conditions, blotted on nitrocellulose, and revealed by incubating the blots with [^{125}I]-labelled streptavidin. We observed that the molecules studied (i.e., N-CAM120, F3, and Thy1) were found both in the cell lysates and in the soluble fraction (Fig. 2). The liberation of GPI-anchored molecules that we observed in the culture medium was not due to damaging the cells by biotinylation as the two transmembranar isoforms of N-CAM (N-CAM140 and 180) were exclusively found in the cell lysate. From this experiment we concluded that N-CAM120, F3, and Thy1 molecules are spontaneously released in the medium from cell membranes; thus, a mechanism should exist that controls their release. However, the heterogeneity of the culture and the possible perturbating effects of biotinylation on this mechanism prevented us from performing quantitative studies on the amounts released. To overcome these difficulties we turned to a cell line expressing these molecules.

Differentiation of the ND26 Cell Line

We recently prepared and characterized immortalized cell lines obtained by fusing postmitotic rat DRG neurons with a mouse neuroblastoma cell line (N18Tg2) [Wood et al., 1990]. We investigated the differentiation state of one of these cell lines, ND26, by manipulating the composition of the culture medium. When cells were cultured in minimum medium whose composition is given in Methods, either alone or containing Bt2cAMP (1 mM), NGF (50 ng/ml) or a mixture of both, we observed that cells did not divide as controlled by estimating cell numbers at various intervals of time. However, addition of Bt2cAMP to the minimum culture medium induced drastic changes in morphology of the cells consisting of an intense neurite outgrowth. NGF has a less intense effect on neurite outgrowth and slower kinetics of growth. These differences were clearly seen after 4 days in culture, when neurite outgrowth was compared in minimum medium by itself (Fig. 3A), and in this medium containing either NGF (Fig. 3B) or Bt2cAMP (Fig. 3C).

To unambiguously establish that these changes in morphology corresponded to a true differentiation towards a more mature neuronal phenotype, we analysed such cultures for their expression of MAP1 (Fig. 4) whose expression is known to be developmentally regulated [Tucker et al., 1988]. After one day of culture in minimum medium containing Bt2cAMP, MAP1 immunoreactivity was confined to cell bodies (Fig. 4A,B) whereas after 4 days in such medium

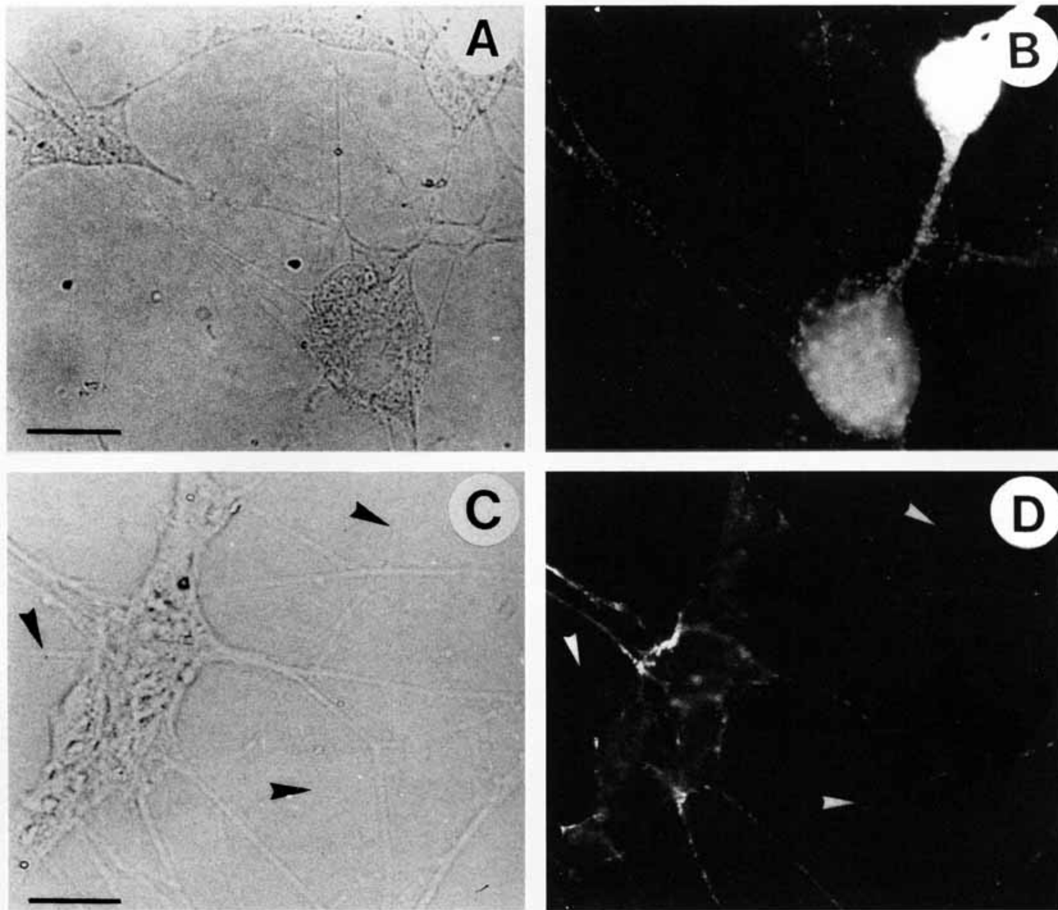

Fig. 1. Expression of GPI-anchored molecules on early postnatal DRG neurons after 3 days of culture in 10% FCS medium supplemented with NGF. Immunofluorescence labelling with polyclonal anti-Thy1 (B) and anti-F3 antibodies (D). A and C are the corresponding phase contrasts. Note that some neurite processes (arrows) are not stained for F3 antibody. Bar = 20 μm.

immunoreactivity was clearly detectable in some processes (Fig. 4C,D). These observations are in good agreement with a differentiation of the cells towards a more mature neuronal phenotype [Nixon et al., 1990].

Expression and Quantitative Analysis of GPI-Anchored Molecules by the ND26 Cell Line

We tested by immunofluorescence staining that the N18Tg2 parental neuroblastoma expresses N-CAM but not Thy1 and F3 [Wood et al., 1990]. These molecules must therefore be encoded by a gene transmitted from the DRG parental cell of the hybrid. For N-CAM the situation was more ambiguous, as we could detect a slight immunoreactivity with the rat anti-N-CAM monoclonal H28 recognizing mouse N-CAM. Nevertheless, a polyclonal anti-N-CAM antibody showed strong staining, suggesting that rat N-CAM is also expressed by the hybrid cell line.

To quantify the expression of GPI-anchored molecules during the differentiation processes without disturbing the physiological state of the cells by chemical treatments such as biotinylation, we set up a slot-blotting procedure that allows a quantitative estimation of the molecules in various compartments. We measured the overall cell content, the quantity of molecules cleavable by PI-PLC enzyme, and spontaneously released molecules in the culture medium. This procedure allowed us to compare several samples under various conditions and to determine the kinetics of the release on the same sample of cells, during differentiation. To this end, cells were cultured in 96-well trays as described in Methods. Culture medium was changed every day and collected; after 2 and 4

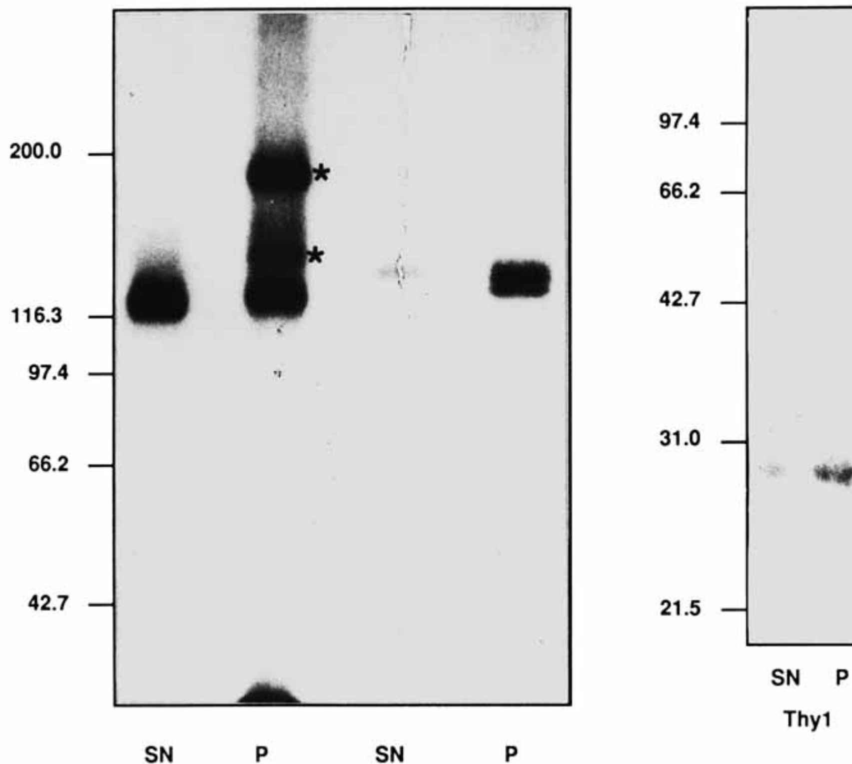

Fig. 2. Spontaneous release of GPI-anchored molecules from DRG neurons. Primary cultures were maintained for 3 days in culture medium supplemented with NGF. Then cells were cell surface biotinylated. After 1 h at 37°C, membrane-bound and spontaneously released products were immunoprecipitated, separated on SDS-polyacrylamide gels, and transferred to Nitroscreen® membranes and detected using [^{125}I]-labelled streptavidin. Polyclonal antibodies directed against N-CAM, F3, and Thy1 molecules were used. SN corresponds to the released molecules after 1 h incubation at 37°C, while P corresponds to membrane-bound molecules. Molecular weights are shown for each blot. Note that N-CAM140 and N-CAM180 (*) are only found in the P fraction, and that no released products were observed in the SN fractions.

days, respectively, cells were treated with PI-PLC enzyme, the medium of cleavage was collected, and the corresponding cells harvested and solubilized in detergent containing buffer. The samples, corresponding to individual wells, were blotted on a nitrocellulose sheet and examined for their content of the tested molecules by incubation with the relevant antibodies. Figure 5 shows examples of results obtained in such slot-blotting experiments with N-CAM and Thy1 molecules, respectively.

Quantitative analysis of such autoradiograms by densitometry or counting of the radioactive slots showed that replacement of FCS containing medium by minimum medium induced a decreased expression of N-CAM (20% between day 2 and day 4). However, our test did not allow us to differentiate between the GPI-anchored and the transmembranar isoforms. By contrast this medium induced the expression of F3 and Thy 1 molecules; moreover, their expression was increased over time in culture (Table I). Therefore, data shown in Table I indicate that suppression of FCS from the medium besides stopping cell division also modifies protein expression without inducing noticeable neurite outgrowth.

Influence of Conditions of Differentiation on GPI-Anchored Molecules Expression

Three conditions of defined medium—namely, addition of NGF, Bt2cAMP, or both to minimum medium—were tested for their influence on GPI-anchored molecules expression. To this end we measured the overall quantity of GPI-anchored molecules after 2 and 4 days of culture in such media. Results are shown in Figure 6 for 4 days of culture. N-CAM did not exhibit any significant change of the levels of expression whatever the medium tested. F3 and Thy1 expression was increased when differentiation was carried out in medium containing Bt2cAMP. This increase was approximately 25% over the values obtained in minimal medium. We also

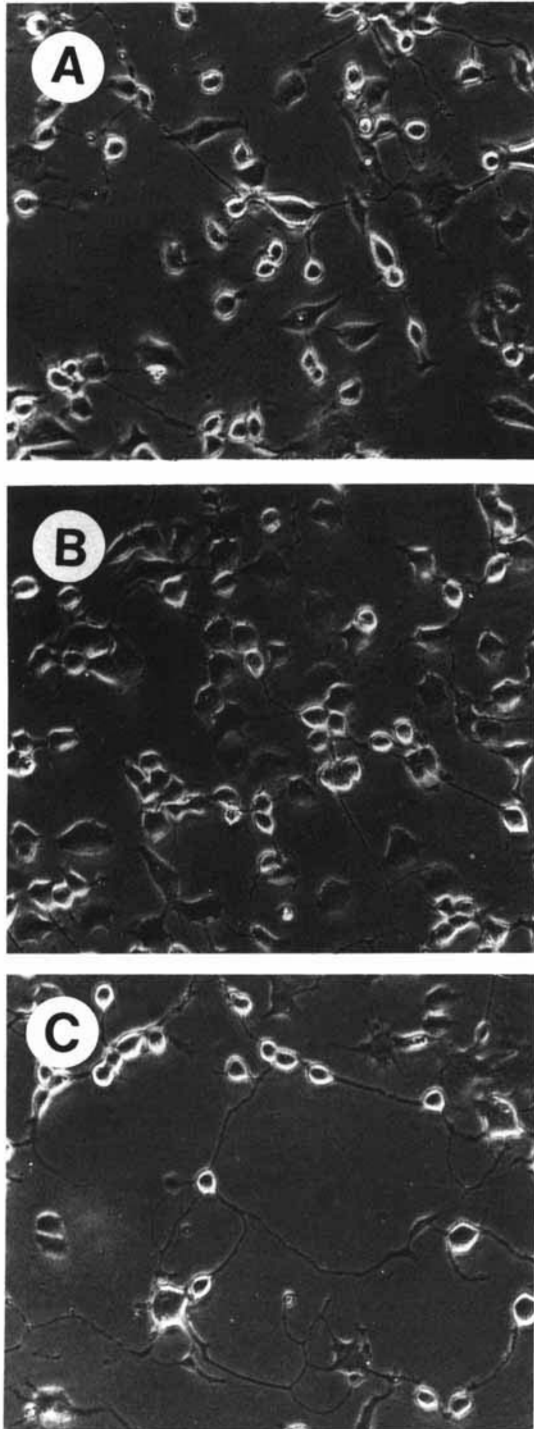

Fig. 3. Cell morphology in various differentiating media. ND26 cells were cultured in three different media for 4 days: minimum medium **(A)**, plus NGF **(B)** or Bt2cAMP **(C)**. In minimum medium (A), some cells grow small neurites, while in the presence of NGF (B) and more particularly Bt2cAMP (C) neurites are longer. In this latter medium outgrowth was more rapid and more intense than with NGF.

observed that NGF alone did not significantly change the level of expression when compared to minimum medium without supplementation.

Quantitative Estimation of PI-PLC Releasable Molecules

Purified PI-PLC from *B. thuringiensis* had been used to selectively remove GPI-anchored proteins from a variety of cell types. However, it has been shown [Roberts et al., 1988; Presky et al., 1990] that some GPI-anchored proteins are resistant to this treatment and that this resistance is variable with the molecule studied or dependent on the cell type expressing it. We analysed this parameter for the various molecules studied on cells maintained either for 2 or 4 days in the four media.

We calculated the percentage of release for each molecule by comparison with the overall quantity expressed by the cells. The percentages were very similar whatever the composition or the state of the cell differentiation (2 or 4 days in Bt2cAMP containing media). Results are shown in Table II for cells maintained for 4 days in Bt2cAMP containing medium. To confirm this result, we also enzyme treated cells after biotinylation of their cell surface and analysed the cleavage medium for its content of released molecules after immunoprecipitation and gel analysis. Comparison of the data obtained from the two approaches showed that the results were similar.

Spontaneous Release of GPI-Anchored Molecules During ND26 Differentiation

We estimated the quantity of spontaneously liberated molecules from a sample of cells maintained in the different defined media. In the 0.2 cm^2 wells the quantities liberated were usually too low to be quantified after 24 hours of culture; thus, we made measurements on deposits corresponding to a pool of medium from 48 hours.

For N-CAM a clear decrease of the release of the GPI-anchored isoform was observed, as the quantity recovered for the first 2 days (taken as 100%) was significantly higher than the quantity released during the 2 following days whatever the medium considered. By contrast, for the two other molecules, the quantity of released molecules increased (Fig. 7A). Thus, the amount of released molecules reflects the amount of molecules expressed by the cells and varied with their differentiation state. Hence, when the overall quantity liberated was com-

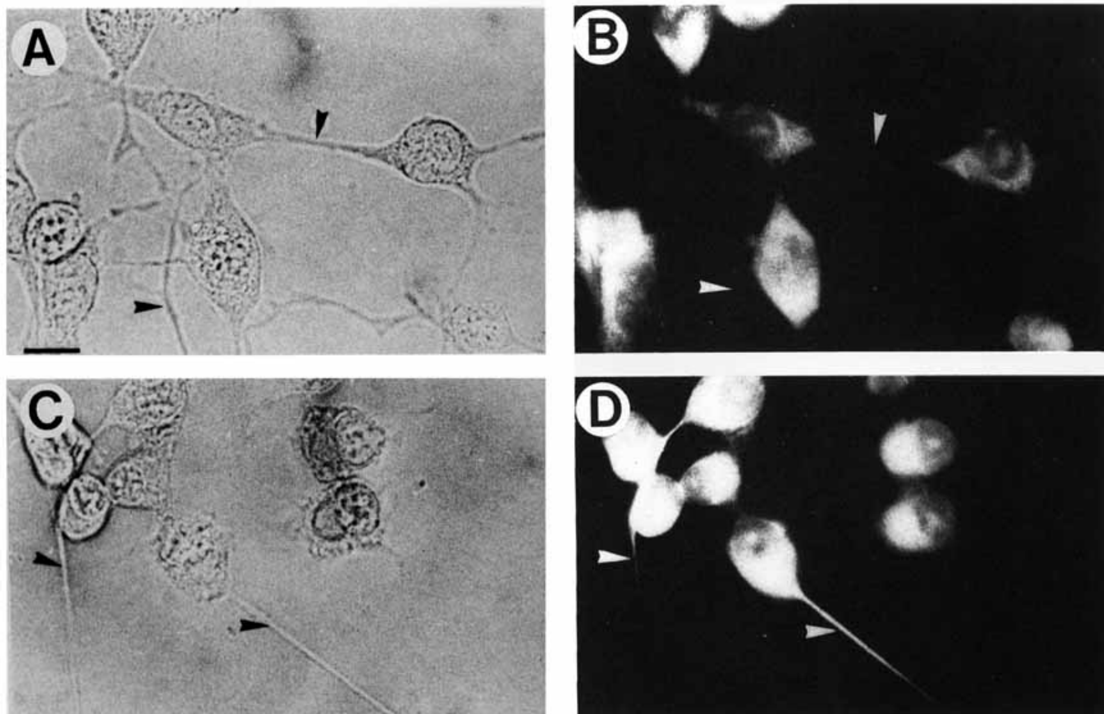

Fig. 4. Immunofluorescence staining for MAP isoforms. Cells were cultured for 1 (**A, B**) or 4 (**C, D**) days in minimum medium containing Bt2cAMP. After one day of culture neuritic extensions are not labelled with anti-MAP1 (arrows in B), while they become labelled after 4 days (arrows in D). Bar = 20 μm.

pared with the total quantities expressed at a given time (i.e., 2 or 4 days in differentiation medium), we observed that the calculated ratio was constant; this is shown for F3 in Fig. 7B, where ratios of 66 and 65% were found, respectively.

DISCUSSION

The first series of observations described in this report have established that early postnatal DRG neurons express N-CAM120 isoform, F3, and Thy1 that are GPI-anchored proteins, members of the immunoglobulin superfamily. These molecules are likely to play a role in cellular recognition processes. Observations by immunofluorescence staining after different times in vitro showed that the expression of these molecules is temporally regulated. F3 and Thy1 became more heavily expressed after 3 days of culture. Such cultures are heterogeneous for the expression of F3, as some neurons do not express it. Moreover, they are dependent for their survival on the presence of NGF.

As already reported for N-CAM120 in cultures of C6 astrocytoma cells [Hé et al., 1987; Théveniau et al., 1991], we observed that all the molecules we studied could be spontaneously released into the culture medium. Biotinylation experiments of the cell surface unambiguously indicated that these liberated molecules originated from the cell surface and were not directly secreted from an intracellular pool.

So far very little is known about the metabolism and regulation of expression of GPI-anchored molecules in general. We have investigated parameters such as expression, accessibility to PI-PLC cleavage, and spontaneous release of such molecules in the hope that this would give insights into their metabolism. Moreover, the study of a neuronal system undergoing differentiation and neurite outgrowth allowed us to investigate whether the parameters studied were dependent upon such phenomena. DRG primary cultures were not well suited for our studies, since it was difficult to obtain adequate numbers of cells and that heterogeneity made biochemical studies difficult.

We therefore used a sensory neuron derived hybridoma termed ND26 (neuroblastoma x DRG neuron hybrid) that we recently generated [Wood et al., 1990]. Here we show that differentiation of this cell line can be controlled by manipulating the composition of the culture medium. Neurite outgrowth was dependent on the presence of Bt2cAMP or to a lesser extent of NGF in the medium. The probing of MAP1 isoform expres-

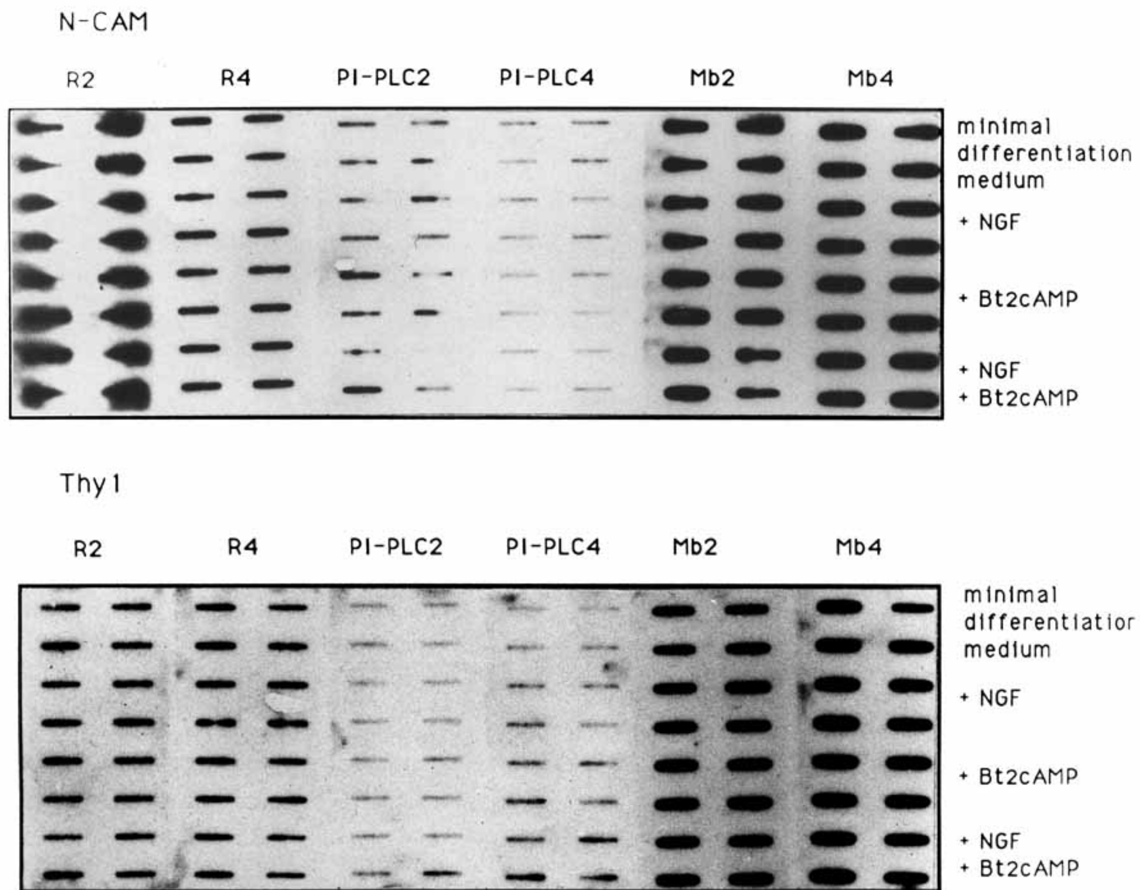

Fig. 5. Immunoslot analysis of GPI-anchored molecules. The same number of cells (15×10^3 cells/well) were seeded in 96-well trays and cultured with 4 different media: minimum medium alone or supplemented with NGF, Bt2cAMP, or both. Culture media were pooled from the first two days (R2) and the following two days (R4). After 2 and 4 days of culture, respectively, half of the wells were treated with PI-PLC. The corresponding media of cleavage are termed PI-PLC2 and PI-PLC4. After PI-PLC treatments, cells were lysed and proteins recovered (Mb2, Mb4) as described in Materials and Methods. Every fraction corresponding to an individual well was slot-blotted onto a nitrocellulose sheet. Each given condition was tested simultaneously on 4 wells. The presence of GPI-anchored molecules was probed by incubation of the nitrocellulose sheet with the corresponding antibody. Bound antibody was revealed by [^{125}I]-labelled protein A. The results for N-CAM and Thy1 molecules are shown. Note that N-CAM expression decreased from 2 to 4 days of culture, while Thy1 expression increased.

sion showed that these morphological changes corresponded to differentiation.

The ND26 cell line retains thus enough features of its sensory neuronal parent to provide a useful model for studies of aspects of GPI-anchored molecules regulation. Moreover, its lack of dependence on NGF for survival allowed us to investigate the effect of this factor on GPI-anchored molecules expression and metabolism in comparison with another differentiating agent, Bt2cAMP, an analogue of the second messenger cAMP [Rydel et al., 1988].

NGF has been shown in vitro to elicit a wide variety of cellular responses, including promoting the survival and morphological differentiation of neurons from embryonic sensory ganglia [Barde, 1980]. In this regard, various groups

TABLE I. Effect of FCS Removal From the Medium on GPI-Anchored Molecules Expression*

	Difference of expression between day 2 and day 4 (%)
N-CAM	−18.7 ± 4.2 (n = 3)
F3	+29.4 ± 2.0 (n = 4)
Thy1	+10.0 ± 5.0 (n = 2)

*Overall expression of molecules was estimated by summing the values obtained in the fractions (culture media + PI-PLC releasable + membranar proteins) at 2 and 4 days, respectively. Cells were maintained in minimum differentiation medium; data (mean ± SEM) gave the difference of expression for individual proteins between day 2 and day 4 (the level of expression at day 2 was taken as the 100% value). n indicates the number of separate experiments performed. Statistical treatment (Duncan's test) showed a significant variation of expression ($P < 0.05$).

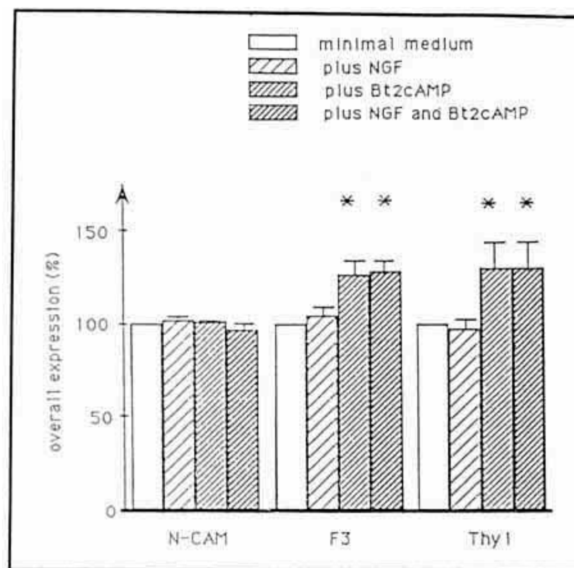

Fig. 6. Overall expression of GPI-anchored molecules after 4 days of culture. Expression of the GPI-anchored molecules was made by a quantitative analysis of the autoradiograms. Data are shown for cells maintained for 4 days in the different media. Overall expression of a given molecule was obtained by summing the values from each compartment—that is, spontaneous release (R2 + R4), releasable by PI-PLC (PI-PLC4), and cell attached (Mb4). Data were analysed using one way treatment analysis of variance, followed by Duncan's multiple range test [1955] for the determination of significant differences between means. The results are expressed as percentages of the expression in the minimum medium. Data are (mean ± SEM) from 3 to 5 experiments. Star indicates a significant difference ($P < 0.01$).

have reported that cAMP analogs and NGF have both synergistic and differential actions on NGF responsive cells [Heidmann et al., 1985]. However, the effect of cAMP analogs and NGF on rat PC12 cell morphology and neurite outgrowth [Riechter-Landsberg and Justorff, 1986] suggests a divergent pathway of action. In our culture conditions when the level of expression of GPI-anchored molecules was monitored in minimum medium supplemented with NGF or Bt2cAMP and compared with levels measured in minimum medium alone, they fall into two groups. The first one includes N-CAM, whose expression was not significantly affected whatever the medium used. The second is comprised of F3 and Thy1 molecules, whose expression increased approximately 25% in presence of Bt2cAMP. This increase could be correlated with the more intense neurite outgrowth that we observed in this medium and the observations made on the involvement of F3 molecules in promoting this process [Gennarini et al., 1991].

TABLE II. Percentage of PI-PLC Releasable Molecules*

	Released molecules (%)
N-CAM120	50.0 ± 5.0 (n = 4)
F3	39.5 ± 6.0 (n = 6)
Thy1	42.4 ± 5.4 (n = 3)

*Amounts of released molecules were estimated by analysis of PI-PLC and Mb fractions on the autoradiograms, except for N-CAM, for which biotinylation experiments were analyzed. The values obtained were compared with the cell content (PI-PLC releasable + membrane values). Data (mean ± SEM) obtained for PI-PLC released molecules are expressed as a percentage of the cell content calculated after 4 days in culture. n indicates the number of separate experiments. No statistically significant differences could be found by comparing the percentage of release for each molecule (Duncan's test).

NGF has been reported to upregulate N-CAM and Thy1 expression in PC12 cells [Prentice et al., 1987; Doherty et al., 1988]. However, control of N-CAM level by NGF does not seem to be a general phenomenon, and NGF had no effect on N-CAM expression by PC12 cells in another study [Friedlander et al., 1986]. ND26 cells were responsive to NGF, since it was able to induce some neurite outgrowth; a possible explanation is that our quantitation test was not sensitive enough to detect minor significant changes.

One of the features of GPI-anchored molecules is that they can be released as a soluble form upon treatment with bacterial PI-PLC enzyme. However, several studies have shown that some GPI-anchored molecules exhibit resistance to this treatment [Low et al., 1988]. An unanswered question is whether this is a general property of all anchored structures expressed by a given cell type, or a property characteristic of each individual molecule, whatever the cell expressing it. Our observations would favor the first hypothesis, as the percentage of molecules resistant to PI-PLC treatment appeared rather constant. The basis for the resistance of GPI molecules to PI-PLC treatment has not been fully explored.

Detailed studies on anchor compositions have shown that structural variations can exist, such as a modification of the inositol rings with fatty acid which renders the molecule insensitive to PI-PLC treatment. This is the case, for example, in human brain acetylcholinesterase. Interestingly, the molecule expressed by bovine erythrocytes does not exhibit the same properties. Data from our laboratory [Théveniau et al., 1991] on N-CAM120 isoform sensitivity to PI-PLC treat-

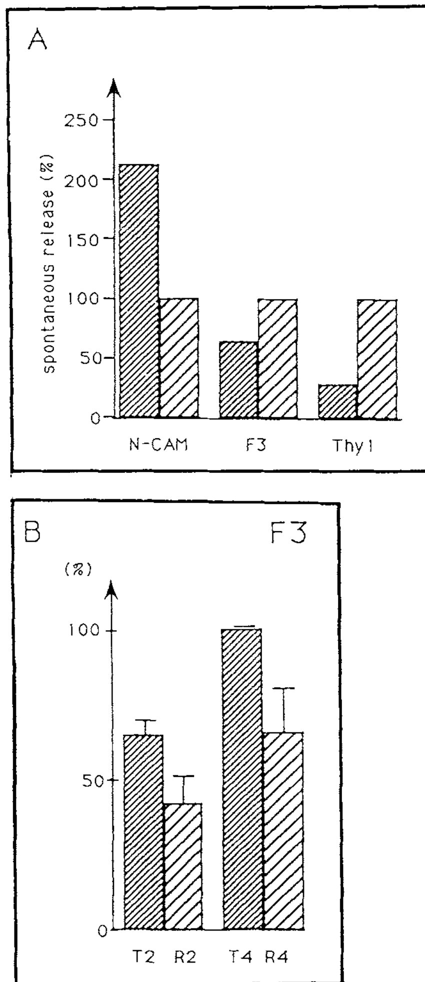

Fig. 7. Spontaneous release of GPI-anchored molecules from ND26 cell line. This is shown for Bt2cAMP medium. **A:** Spontaneous release was estimated by quantification of R2 and R4 spots on the autoradiograms. Values obtained for R4 were taken as 100%. Note that spontaneous release increased for every molecule except N-CAM, for which we measured a decrease. The differences measured were significant. **B:** Spontaneous release of F3 protein analysed after 2 and 4 days of culture (R2 and R4) by comparison with its overall expression at day 2 (T2) and day 4 (T4). The expression of F3 increased during differentiation, but the ratios R2/T2 and R4/T4 are constant.

ment showed that it was almost (>95%) completely releasable from the C6 astrocytoma cell membrane. Other studies on the role of GPI-anchored proteins on T cells [Presky et al., 1990] showed that T cell activation led to a marked decrease in the ability of PI-PLC to remove GPI-anchored surface proteins from activated cells. Taken together these observations suggest that the sensitivity of GPI-anchors to PI-PLC is regulated by the cell. Moreover, as in the differentiating ND26 cell line, the percentage of releasable molecules is rather similar for all the molecules we studied; this suggests that there is a common mechanism of control for the anchors of GPI-linked proteins.

All molecules were shown to be spontaneously released into the medium. If such molecules have a different role from their membrane counterpart, we may expect this release to be regulated. For each studied protein a clear correlation was observed between the overall amount expressed by the cells and the quantity released. These data are intriguing and are in favor of a shared mechanism of release. The amount of released molecules would not be individually modulated by mechanisms taking place at the cell surface, but rather controlled by the level of expression of each individual molecule. This does not preclude a role for the soluble form in controlling the level of expression of the considered molecule by an autocrine mechanism of regulation of synthesis [Roubin et al., 1990], and further experiments are needed to address this issue. Alternatively, we cannot exclude the possibility that modulation of release can occur via extrinsic factors such as surface phospholipase C of neighbouring cells as suggested by Furley et al. [1990]. Our system is not well suited to detect this possibility, as the culture is homogenous, and fasciculation is not observed.

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