Polysialic acid (PSA) is a regulatory epitope of neural cell adhesion molecule (NCAM) in homophilic adhesion of neural cells mediated by NCAM, is also known to be re-expressed in several human tumors, thus serves as an oncodevelopmental antigen. In this study, using a recently developed ultrasensitive chemical method in addition to immunochemical methods, growth stage-dependent and retinoic acid (RA)-induced differentiation-dependent changes of PSA expression in human neuroblastoma (IMR-32) and rat pheochromocytoma (PC-12) cells were analyzed both qualitatively and quantitatively. Both IMR-32 and PC-12 cells expressed PSA on NCAM, and the level of PSA expressed per unit weight of cells increased with post-inoculation incubation time. The most prominent feature was seen at the full confluence stage. RA induced neuronal differentiation in both IMR-32 and CP-12 cells that paralleled the change in the PSA level. Chemical analysis revealed the presence of NCAM glycoforms differing in the degree of polymerization (DP) of oligo/polysialyl chains, whose DP was smaller than 40. DP distribution of PSA was different between the cell lines and was changed by the growth stage and the RA treatment. Thus DP analysis of PSA is important in understanding both mechanism and biological significance of its regulated expression.
to neuronal differentiation (13). This report represents the first qualitative and quantitative analysis of PSA expression during neuronal differentiation of PSA-positive cell lines (IMR-32 and PC-12). These have proven to be good in vitro models for studying how changes in the PSA expression would result in fine-tuning of NCAM-mediated cell-cell association.

**EXPERIMENTAL PROCEDURES**

**Antibodies Used**—Rat anti-mouse embryonic PSA-NCAM (CD56) mAb (12F8, IgM) was purchased from BD Pharmingen. Anti-human NCAM mAb (VIN-IS-53, IgG) developed by P. W. Andrews, and anti-rat NCAM mAb (5B8, IgG), developed by T. M. Jessell and J. Dodd, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA.

**Cell Lines and Their Culture and Harvest**—We used the following cell lines to examine the expression of PSA on NCAM: human neuroblastoma (IMR-32), human neuroblastoma from metastatic site of bone marrow (SK-N-Sh), human neuroblastoma from metastatic site of supra-orbital area (SK-N-MC), mouse neuroblastoma (neuro-2A), and rat pheochromocytoma (PC-12). All of these cell lines were obtained from the American Type Culture Collection (ATCC) and were purchased from Food Industry Research and Development Institute, Hsinchu, Taiwan. Each cell line was cultured in minimal essential medium to a final concentration of 10^5/ml and were collected at different stages after inoculation and incubation at 37 °C for 0.5–1 h with 100 μl of 0.5% Triton X-100. The insoluble material was removed by centrifugation, and the supernatant was used for analysis. The cells with and without RA treatment were analyzed using the same procedure.

**Analysis of PSA and Total Neu5Ac in the Solubilized Glycoproteins**—PSA and total Neu5Ac in the glycoproteins solubilized with Triton X-100 were analyzed by HPLC-FD after derivatization with DMB (Neu5Ac)\_\_Q, DMB-tagged ε2-8-linked poly(Neu5Ac) with DP N that is obtained by reacting (Neu5Ac\_\_\_ with DMB, where Q denotes fluorescent chromophore, quinoxalinone derivative) (7–9). The DMB reagent contained 5.4 mM DMB (Dojinbo, Kumamoto, Japan), 18 mM sodium hydrosulfite, 1 mM β-mercaptoethanol, and 40 mM trifluoroacetic acid. For PSA analysis, a 40-μl portion of the solubilized fraction was mixed with 40 μl of the DMB reagent. The mixture was incubated for 48 h at 10 °C. After the reaction, 20 μl of 1 N NaOH was added to the ice-cold reaction mixture to hydrolyze lactones formed during incubation under acidic conditions. The alkalized reaction mixture was left for 30 min at room temperature and centrifuged, and an 80-μl portion of the supernatant was injected on a DNAPEc PA-100 column (Dionex, Sunnyvale, CA). The elution condition was described previously (7–9). Peaks of oligo/poly(Sia) peaks (DP >4) represents the total number of PSA chains and used as a value comparing the level of PSA in the cells.

**ELISA** was performed as a method to evaluate quantitatively the level of PSA in the cells. A 50-μl portion of the solubilized membrane-bound glycoprotein was dried in a well of a 96-well plastic plate and subjected to ELISA using mAb 12F8 and alkaline phosphatase-conjugated goat anti-rat IgM, as the primary and secondary antibody, respectively. The absorbance at 405 nm (p-nitrophenol) of the reaction mixture represents the amount of PSA epitope.

For total glycoprotein-bound Neu5Ac analysis, a 4-μl portion of the solubilized samples from delipidated cell-extracts was hydrolyzed in 80 μl of a final concentration of 10 μl. Cells were inoculated, cultured, and harvested as described above and were used for analysis. The cells at different stages of development were examined for morphological changes under a phase-contrast microscope.

**Processing of Cells for the Analysis of PSA and Total Glycoprotein-bound Neu5Ac**—Methods were similar to those described previously (9). In brief, cells (100 mg, wet weight) were homogenized in a Polytron homogenizer (Kinematica, Litan, Switzerland) in 600 μl of chloroform, methanol, 0.01 M Tris-HCl (pH 8.0) mixture (4:8:3, v/v/v). The residue collected by centrifugation was finally washed with cold 80% ethanol. Glycosylated and non-glycosylated proteins were solubilized by sonication and incubation at 37 °C for 0.5–1 h with 100 μl of 0.5% Triton X-100. The insoluble material was removed by centrifugation, and the supernatant was used for analysis. The cells with and without RA treatment were analyzed using the same procedure.

**TABLE I**

Diagnosis for PSA and NCAM in four different neuroblastoma cell lines (IMR-32, SK-N-MC, SK-N-SH, and Neuro-2A) and pheochromocytoma cell line (PC-12) by DMB/HPLC-FD and by Western blot analysis using anti-PSA mAb (12F8) and anti-NCAM mAbs (5B8 and VIN-IS-53), together with the data for the amount of glycoprotein-bound Neu5Ac.

|                         | PSA by DMB/ HPLC-FD method | Western blot | Glycoprotein-bound Neu5Ac μg/100 mg wet cells |
|-------------------------|-----------------------------|--------------|---------------------------------------------|
|                         | Anti-PSA mAb, 12F8          | Anti-NCAM mAb, 5B8 | Anti-NCAM mAb, VIN-IS-53 |                                |
| Human neuroblastoma, IMR-32 | +                          | +             | +                                           | 3.0                          |
| Human neuroblastoma, SK-N-MC | –                          | –             | –                                           | 0.41                         |
| Human neuroblastoma, SK-N-SH | –                          | –             | –                                           | 0.25                         |
| Mouse neuroblastoma, Neuro-2A | –                          | –             | –                                           | 0.38                         |
| Rat pheochromocytoma, PC-12 | +                          | +             | –                                           | 2.2                          |

**FIG. 1.** Western blot analysis of the changes in PSA expression, using anti-PSA antibody in IMR-32 (A) and PC-12 cells (B) during cell culture for 24 (lane a), 36 (lane b), 48 (lane c), 60 (lane d), 72 (lane e), and 84 h (lane f) (full confluence). A detergent extract from the cells at each stage (10 μg of protein) was subjected to SDS-PAGE, blotted on polyvinylidene difluoride membrane, and immunostained with the anti-PSA mAb, 12F8 (CD56).
Evaluation of PSA in IMR-32 and PC-12 Cells

FIG. 2. PSA analysis by DMB/HPLC-FD in IMR-32 (A) and PC-12 cells (B) cultured for 24 (a), 36 (b), 48 (c), 60 (d), 72 (e), and 84 h (f) (full confluence). Membrane-bound glycoproteins from 100 mg of wet cells at each stage were solubilized and derivatized with DMB as described under “Experimental Procedures.” Each sample containing 130–950 ng of total Neu5Ac (derived from 32 mg of cells) was injected into a DNAPac PA-100 column and eluted with 0.02–0.35 M NaNO₃, and the elution was monitored by fluorescence intensity. Peaks were labeled with the DP values.
Evaluation of PSA in IMR-32 and PC-12 Cells

Fig. 2—continued
RESULTS

Diagnostic Examination for the PSA and NCAM Expression in Various Cell Lines of Neuronal Origin—The amount of Neu5Ac and the expression of PSA and NCAM in the solubilized glycoproteins were analyzed for 3 human and 1 mouse neuroblastoma cell lines and a rat pheochromocytoma cell line, all harvested at full confluence stages. The results summarized in Table I demonstrate that two methods (chemical and immunochromatography) led to the same conclusion that IMR-32 and PC-12 express PSA. These results are partly in conflict with the previous report (21) showing the absence of PSA in NCAM-positive wild type PC-12. Both of these cell lines were NCAM-positive, and immunostaining of the gel after SDS-PAGE using either anti-PSA or anti-NCAM antibody showed a similar pattern indicating that PSA was expressed on NCAM. It is noted that mAb VIN-IS-53 is strictly specific for human NCAM polypeptide sequence. It is also noted that the values of total glycoprotein-bound Neu5Ac determined by the present method showed 1 order of magnitude increase in PSA-positive IMR-32 and PC-12 compared with PSA-negative cells, indicating that we can estimate the possible presence of PSA and evaluate its level from these values.

Expression and Change in the Level of Polysialylated NCAM in IMR-32 and PC-12 Cells at Different Growth Stages as Revealed by Immunoreactivity against Anti-PSA mAb (12F8) and DMB/HPLC-FD Method—Neuroblastoma cells are known to undergo differentiation into neuronal cells by various chemical agents such as retinoic acid (RA) (12). We examined the effects of RA added during cell culture on PSA expression in both IMR-32 and PC-12 cells. The PSA expression in RA-treated cells harvested at different culture times was analyzed by Western blot analysis using monoclonal antibodies, 12F8 and 5BS. The mAb 12F8 recognizes the PSA portion of polysialylated NCAM, and mAb 5BS reacts with the polysialic acid portion common to three major isoforms with molecular masses of 180, 140, and 120 kDa (16). In both IMR-32 and PC-12 cells relatively narrow bands were immunostained with anti-PSA centered at about 150,000 were seen (Fig. 1, A and B). Immunostaining with anti-NCAM showed similar patterns indicating that PSA was exclusively expressed on NCAM (data not shown). The results also indicated that the 140-kDa NCAM was the major isoform in these cells. Immunostained smears obtained at each culture time showed that in both cell lines the expression level of PSA was small during the initial 24 h and increased with growth stage between 36 and 84 h when the cells attained confluence. It is noted that in PC-12 the smears are larger than in IMR-32 cells, suggesting the level of PSA-NCAM is higher in PC-12 than in IMR-32 (see below).

Analysis of PSA Expression in IMR-32 and PC-12 Cells at Different Growth Stages by the DMB/HPLC-FD Method—We have established recently a highly sensitive and reliable method of PSA analysis (DMB/HPLC-FD). The outstanding feature of the method is in the presence of PSA, and its DP distributions are analyzed simultaneously by using only as small amount a sample as used for immunochromatographic methods. In this study the sample injected to the HPLC column was derived from 32 mg of wet cell, whereas samples loaded to 1 lane in SDS-PAGE or added to a well in ELISA were derived from 6 to 16 mg and 50 mg of wet cells, respectively.

The results of DMB/HPLC-FD analysis for the cells harvested at different stages (24, 36, 48, 60, 72, and 84 h following inoculation) are reproduced in Fig. 2, A and B, for IMR-32 and PC-12 cells, respectively. The appearance of typical ladder-like peaks assignable to DMB-labeled oligo/poly( Neu5Ac) indicates the presence of PSA in these cells at all growth stages. The total area of the ladder-like peaks represents the total number of PSA chains. By analyzing the same amount of the different cell lines, the level of PSA between the cell lines can be compared. As can be seen in Fig. 2, A and B (a–f), the amount of PSA expressed in both IMR-32 and PC-12 cells increased with culture time, and is most prominent at the full confluence stage (84 h). The growth stage-dependent change in PSA expression can be viewed more quantitatively by bar graphs showing the total peak areas in DMB/HPLC-FD as well as the PSA levels estimated by ELISA and the amount of total protein-bound Neu5Ac (mg/mg of wet cells) (Fig. 3, A and B, for IMR-32 and PC-12, respectively). Regardless of the method used, we can conclude that the PSA level in both cell types increased with cell culture time and reached the maximum values at confluence stages (i.e., 84 h culture; after this stage the number of dead cells increased). In both cell lines, there is some disagreement between the PSA level estimated by the DMB/HPLC-FD method and that estimated by ELISA. This discrepancy may be ascribed to the fact that DMB/HPLC-FD determined the number of PSA chains, whereas ELISA quantified the number of PSA epitope bound to the antibody. Results obtained from Western blot analysis were similar to those obtained from ELISA.

Expression and Change in the Level of Polysialylated NCAM in IMR-32 and PC-12 Cells Cultured in the Presence of Retinoic Acid as Evaluated by Reactivity against Anti-PSA mAb (12F8) and DMB/HPLC-FD Method—Neuroblastoma cells are known to undergo differentiation into neuronal cells by various chemical agents such as retinoic acid (RA) (12). We examined the effects of RA added during cell culture on PSA expression in both IMR-32 and PC-12 cells. The PSA expression in RA-treated cells harvested at different culture times was analyzed by Western blot using anti-PSA (Fig. 4, A and B). Immunostained smear bands showed quite different patterns between IMR-32 and PC-12 cells. A relatively large smear shown by IMR-32 appearing after the initial 24 h suggests a relatively high level of PSA-NCAM. The smear size slightly increased until 72 h but decreased to almost undetectable levels after 84 h. By contrast, in PC-12 the level of PSA-NCAM was low during the initial stage but greatly increased in the next 24 h to attain the maximum value after 48 h. It then gradually decreased and reached the initial low value after 84 h of culture. The results of PSA analysis by the DMB/HPLC-FD method in the cells cultured in the presence of RA are depicted in Fig. 5, A (IMR-32) and B (PC-12). Comparing Fig. 5A with Fig. 2A, we can
conclude that throughout all culture stages until the confluence stage (60 h), differentiated IMR-32 cells synthesized larger numbers of PSA chains than the undifferentiated cells. In RA-treated PC-12 cells, there was no large increase in the number of PSA chains as in the untreated cells (cf. Figs. 2B and 5B). In contrast, ELISA analysis revealed a rapid increase in the PSA epitope in RA-treated PC-12, reaching the maximum value after 48 h, and then gradually decreased. The change in the number of PSA chains in RA-treated cells at different stages quantified by the DMB/HPLC-FD was shown as the bar graphs in Fig. 6, A (IMR-32) and B (PC-12). The change in the number of PSA epitopes estimated by ELISA using anti-PSA antibody and the amounts of total glycoprotein-bound Neu5Ac were also shown in the bar graphs (Fig. 6, A and B). Again there is disagreement between the number of PSA chains and that of PSA epitopes.

Analysis of DP of PSA Chains Expressed in IMR-32 and PC-12 Cells Cultured in the Absence and Presence of RA—We confirmed that DMB/HPLC-FD is an excellent method to determine the DP of PSA (7–9). We analyzed the distribution of DP in the PSA chains in IMR-32 and PC-12 cells at different stages cultured in the absence and presence of RA, and the results are depicted in Fig. 7, A (for IMR-32) and B (for PC-12). A more prominent difference between the cell lines was observed in undifferentiated cells. IMR-32 produced higher DP than PC-12 (compare Fig. 7, A, a, and B, a). Preferential occurrence of high DP PSA was observed in undifferentiated IMR-32 cells cultured for 60 h (Fig. 7A, a). The DP distribution pattern observed in IMR-32 cells at the 60-h stage was similar to that observed for embryonic day 14 chicken brain NCAM (DP 5–10, 31%; DP 11–20, 32%; DP 21–30, 24%; and DP >30, 13%) when the highest level of PSA was synthesized. Although in embryonic chicken brain, PSA of DP >40 occurred at 2% of the total peak area, the cultured cells hardly showed PSA of DP >40. It should be noted that in these analyses the total peak areas for the chicken brain sample and the IMR-32 sample were almost the same, indicating that the appearance of high DP did not result from a large injected amount. It should also be noted that the preferential occurrence of high DP in IMR-32 cells was observed at the stage when the number of PSA chains was about 30% of the maximum expression. RA treatment caused decreased expression of high DP (>30) PSA chains in IMR-32 (cf. Fig. 7A, a and b) but no observable changes in PC-12 (cf. Fig. 7B, a and b). The difference in DP and DP distribution of PSA chains cannot be analyzed by any existing immunochromatographic methods.

Change in Morphology and Cellular Adhesive Behavior of IMR-32 and PC-12 Cells With and Without RA Treatment—PC-12 cells were reported to express two major isoforms of NCAM (NCAM180 and NCAM140) and undergo neuronal differentiation, e.g. neurite formation, in response to nerve growth factor (NGF) (17). In this study the effects of RA on neurite outgrowth and cellular adhesive properties were examined in cultured IMR-32 and PC-12 cells. When IMR-32 cells were incubated in the absence of RA, cells formed aggregates at the initial stages (Fig. 8A, a) and gradually underwent dissociation of cell aggregates at their later stages (Fig. 8A, b–f). These changes were associated with a parallel increase in the level of PSA per unit weight of wet cells (see above). In sharp contrast to these observations, in the presence of 10 μM RA, IMR-32 cells did not form aggregates at the initial stages of incubation, and more than 80% of the cells differentiated to a neuronal phenotype within a day (Fig. 8B, a). They extended long neuritic processes during incubation (Fig. 8B, a–e), and finally after 84 h of culture a large portion of the cells formed large aggregates (Fig. 8B, f). Similar morphological changes were observed for PC-12 cells during growth in the presence of RA (cf. Fig. 9, A and B). Because cell aggregation can be correlated to the change in the properties of cell surface molecules including NCAM, the expression level of PSA must be considered one factor influencing the ability of cells to aggregate.
FIG. 5. PSA analysis by DMB/HPLC-FD in IMR-32 (A) and PC-12 cells (B) cultured in the presence of retinoic acid for 24 (a), 36 (b), 48 (c), 60 (d), 72 (e), and 84 h (f). Membrane-bound glycoproteins from 100 mg of wet cells at each stage were subjected to DMB/HPLC-FD analysis as described in Fig. 2. Peaks are labeled with the DP values.
Evaluation of PSA in IMR-32 and PC-12 Cells

Fig. 5—continued
DISCUSSION

We have examined the expression of PSA and NCAM in several human and mouse neuroblastoma and rat pheochromocytoma (PC-12) cell lines by the recently developed ultrasensitive chemical method (DMB/HPLC-FD) and also by the Western blot method using anti-PSA and anti-NCAM monoclonal antibodies as specific immunochemical diagnostic probes. IMR-32 and PC-12 were PSA-positive/NCAM-positive; neuro-2A was PSA-negative/NCAM-positive, and SK-N-MC and SK-N-SH were PSA-negative/NCAM-negative. The overall conclusion was that NCAM molecule does not occur on all neuroblastoma cells, and PSA is not expressed on all NCAM immunoreactive cells.

Although neuronal cell type neuro-2A has been widely used as a good in vitro model for studying neuronal differentiation, apoptotic cell death, and molecules modulating these processes, there are only a very limited number of studies on PSA expression and enzymes responsible for the synthesis of PSA chains (18–20). Previous studies (18) have yielded conflicting results with respect to expression of PSA in neuro-2A. We first examined neuro-2A as a potential model system for studying detailed structure-function relationships of PSA chains on NCAM, but expression of PSA in neuro-2A cells was not detected by chemical and immunochemical methods. During in vitro neuronal differentiation of mouse embryonal carcinoma P19 cells, PSA was found not to be expressed on undifferentiated cells (day 0) or cell aggregates (days 1–3) but was induced with RA (21). Expression of PSA began after cell aggregates had been dissociated and re-plated on a dish (day 4) and increased up to day 7. The expression of ST8SiaII gene was negligible in both undifferentiated and aggregated cells, but began at day 4, after RA induction, and dramatically increased to reach the maximum level at days 6–7. In this study, neuro-2A cells were analyzed for PSA also under treatment with RA, but no sign of PSA expression was found.

Therefore, we selected two cell lines, IMR-32 and PC-12, to characterize their NCAM and PSA expression and morphology in the absence and presence of RA. Some conflicting reports were also found with respect to the PSA expression in rat PC-12 cells. (i) In 1983 Margolis and Margolis (22) reported the possible presence of PSA in the highly negatively charged glycopeptides derived from PC-12 cells. (ii) Livingston et al. (10) reported the possible presence of an endogenous PSA because a membrane fraction of PC-12 cells acted as an endogenous acceptor of [14C]Neu5Ac in an Escherichia coli K1 sialyltransferase assay. (iii) The calcium-independent protein kinase Cδ

Fig. 6. Comparison of PSA levels in IMR-32 (A) and PC-12 cells (B) at different culture times in the presence of RA. The levels of PSA were evaluated by the three different methods as described in Fig. 3.

Fig. 7. DP analysis of PSA chains expressed in IMR-32 (A) and PC-12 cells (B) in the absence (a) and in the presence (b) of RA by the DMB/HPLC-FD method. DP distribution was evaluated by the sum of peak areas of the DMB-oligo/poly(Sia) peaks in each DP range and expressed as percentage of total.
isozyme was reported to be inversely related to NCAM polysialylation state in rat PC-12 cell line (18). (iv) Horstkorte et al. (23) reported that PC-12 cells only produce a stable polysialylated NCAM when transfected with ST8SiaIV.

Until now two techniques were usually employed to evaluate the amount of PSA expression and DP of PSA chains as follows: one is ELISA and the other is assessment of the intensity and migration rate of smear bands on Western blot. In a recent study of RA-induced changes in PST (ST8SiaII and ST8SiaIV) mRNA expression level and NCAM polysialylation of human neuroblastoma (SH-SY5Y and LAN-5) cells, the increased expression of ST8SiaIV was shown to be closely related to an accelerated polysialylation of NCAM estimated by ELISA (13), although it remains to be determined if there exists any difference in DP of PSA chains produced by ST8SiaII and ST8SiaIV.

In this study three methods were used to assess the expression level of PSA during growth and differentiation of neuroblastoma and pheochromocytoma cells: (i) DMB/HPLC-FD analysis (total peak area measurements); (ii) ELISA; and (iii) determination of total protein-bound Neu5Ac (Figs. 3 and 6). The results provide the first quantitative analyses of changes in the NCAM polysialylation level during cell growth and differentiation. The three methods were used to compare the expression level of PSA during cell growth before and after RA treatment (Figs. 3 and 6). We noted significant disagreement in the PSA levels estimated by DMB/HPLC-FD and ELISA methods for some samples. This may be partly because DP distribution patterns of PSA chains expressed on a variety of NCAM glycoforms are not simple, and anti-PSA antibody could not accurately quantify PSA, as we have repeatedly emphasized (6–9). The antigen specificity resides in the DP of PSA chains, and oligomers as low as DP 3 and 4 can be recognized by some

Fig. 8. Morphological changes of IMR-32 cells cultured in the absence (A) and in the presence (B) of RA, for 24 (a), 36 (b), 48 (c), 60 (d), 72 (e), and 84 h (f). Cells at each stage were examined under a phase-contrast microscope.
anti-PSA antibodies. It is also uncertain at this moment how many antibody molecules can bind to NCAM glycoforms having larger PSA chains (DP ≥ 20). We can thus conclude that the discrepancy in the values obtained by either chemical or immunochemical methods is ascribable to the nature of their reactions. The DMB/HPLC-FD method appears to be superior to the immunochemical methods because it provides information on the DP of PSA chains. Unfortunately, in a number of recent papers (12, 13) reporting studies of the correlation between polysialyltransferase mRNA expression and NCAM polysialylation, the estimation of PSA was based on PSA immunoreactivity, and they may not depict entirely accurate information.

Precise information not only on DP of PSA chains on NCAM but also on their distribution is considered important in understanding (i) the molecular mechanism of biosynthesis of PSA chains, i.e., correlation of poly(ST) with DP of PSA, (ii) fine-tuning of NCAM-NCAM adhesive interaction by PSA chains on NCAM glycoforms, and (iii) correlation of PSA chain length and the metastatic potential of neuroblastoma cells or more in general tumor progression. Some estimates of PSA chain lengths were done by the elution positions on anion-exchange chromatography of PSA-containing glycopeptides derived from PSA-NCAM (24, 25). DP values thus obtained may be significantly overestimated because sulfate groups were shown to be present on the core glycan chains (22, 26–28). Moreover, this method also suffers from the fact that it measures the total numbers of Sia and sulfate residues per N-glycan chain. In

**Fig. 9.** Morphological changes of PC-12 cells cultured in the absence (A) and in the presence (B) of RA for 24 (a), 36 (b), 48 (c), 60 (d), 72 (e), and 84 h (f). Cells at each stage were examined under a phase-contrast microscope.
addition, a possible attachment of PSA chains on the two antennae of a single core N-glycan should be taken into account (26). We believe that the DMB/HPLC-FD method will alleviate these shortcomings in future studies in these areas.

The goals of our present line of study are as follows: (i) to characterize a divergent number of NCAM glycoforms differing in the DP of PSA expressed in different cell types under different physiological conditions; (ii) to correlate invasive growth and metastatic potential, if any, of NCAM-expressing tumor cells and NCAM glycoforms with varying DP values, and (iii) to determine how a large number of such NCAM glycoforms regulate differentially the strength of cell-cell and cell-substratum adhesive interaction mediated by NCAM. In particular, concerning an as yet unanswered question related to the biosynthesis of PSA chains on NCAM, it is still uncertain how the levels and DP distribution of the PSA chains are regulated at different stages of development. Although it has become certain that two polysialyltransferases, ST8SiaIV and ST8SiaII, are involved in catalysis of polysialylation of NCAM (24, 25, 29–38), it still remains unanswered what is the specific role of each of the two closely related polysialyltransferases. No clear data are available to answer the basic question as to whether there exists any difference in DP value of poly(Sia) chains formed by ST8SiaIV and ST8SiaII.

Many forces need to be considered in order to consider the sources of counter-adhesive properties of PSA in NCAM. As cell membranes are brought together they experience several types of forces such as van der Waals attraction, electrostatic attraction/repulsion, hydration repulsion, and specific charge-charge interactions. Counter-adhesive processes in PSA-NCAM may predominantly involve steric hindrance (hydration repulsion) (39) and, perhaps less significantly, charge repulsion of PSA chains. Our results strongly suggest that PSA chains with DP values of 20–30 appear to be long enough to inhibit homophilic adhesion of NCAM. PSA chains of more than DP 30 occur much less frequently, if ever, and such long PSA chains may not be necessary to prevent aggregation of neural cells. In the present study, the largest DP of PSA on NCAM by DMB/HPLC-FD was 45 (this method permits us to detect PSA up to DP 90 when colominic acid was analyzed). These results lead us to question whether high DP PSA chains (DP >40), which occur on only a tiny proportion of NCAM molecules at certain stages of growth and differentiation, are of biological significance.

REFERENCES

1. Inoue, Y., and Inoue, S. (1999) Pure Appl. Chem. 71, 789–800
2. Krushel, L. A., Prieto, A. L., Cunningham, B. A., and Edelman, G. M. (1993) Neuroscience 53, 797–812
3. Roth, J., Zuber, C., Taatsjes, D. J., Blaha, I., and Heitz, P. U. (1989) Verh. Dtsch. Ges. Pathol. 73, 372–387
4. Lin, S.-L., Inoue, Y., and Inoue, S. (1999) Glycobiology 9, 807–814
5. Lin, S.-L., Inoue, S., and Inoue, Y. (2000) Carbohydr. Res. 329, 447–451
6. Inoue, S., Lin, S.-L., and Inoue, Y. (2000) J. Biol. Chem. 275, 29968–29979
7. Inoue, S., and Inoue, Y. (2001) J. Biol. Chem. 276, 31863–31870
8. Inoue, S., Lin, S.-L., Lee, Y. C., and Inoue, Y. (2001) Glycobiology 11, 759–767
9. Inoue, S., and Inoue, Y. (2001) Biochimie (Paris) 83, 605–613
10. Livingston, B. D., Jacobs, J. L., Glick, M. C., and Troy, F. A. (1988) J. Biol. Chem. 263, 9443–9448
11. Rougon, G. (1993) Eur. J. Cell Biol. 61, 197–207
12. Cervello, M., D’Amelio, L., Tesoro, V., Rougon, G., and Matranga, V. (1997) Eur. J. Cell Biol. 73, 270–275
13. Seidenfaden, R., and Hildebrandt, H. (2001) J. Neurobiol. 46, 11–28
14. Inoue, S., Lin, S.-L., Chang, T., Wu, S.-H., Yao, C.-W., Chau, T.-Y., Troy, F. A., and Inoue, Y. (1998) J. Biol. Chem. 273, 27199–27204
15. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
16. Edelman, G. M., and Chuong, C. M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7036–7040
17. Park, T. U., Lucka, L., Reutter, W., and Horstkorte, R. (1997) Biochem. Biophys. Res. Commun. 234, 686–689
18. Gallagher, H. C., Odumera, O. A., and Regan, C. M. (2000) J. Neurosci. Res. 61, 636–645
19. Bojic, U., Ehlers, K., Ellerbeck, U., Bacon, C. L., O’Driscoll, E., O’Connell, C., Berezin, V., Kawka, A., Lepekhin, E., Bock, E., Regan, C. M., and Nas, H. (1998) Eur. J. Pharmacol. 354, 289–299
20. Breen, R. C., and Ronayne, E. (1998) Neuroreport 9, 970–972
21. Kojima, N., Kono, M., Yoshida, Y., Tachida, Y., Nakafuku, M., and Tsuji, S. (1996) J. Biol. Chem. 271, 22058–22062
22. Margolis, R. K., and Margolis, R. U. (1983) Biochem. Biophys. Res. Commun. 116, 889–894
23. Horstkorte, R., Lessner, N., Gerardy-Schahn, R., Lucka, L., Danker, K., and Reutter, W. (1999) Exp. Cell Res. 246, 122–128
24. Angata, K., Suzuki, M., and Fukuda, M. (1998) J. Biol. Chem. 273, 28524–28532
25. Angata, K., Suzuki, M., McAuliffe, J., Ding, Y., Hindsgaul, O., and Fukuda, M. (2000) J. Biol. Chem. 275, 18594–18601
26. Kudo, M., Kitajima, K., Inoue, S., Shiokawa, K., Morris, H. R., Dell, A., and Inoue, Y. (1996) J. Biol. Chem. 271, 32667–32677
27. Liedtke, S., Geyer, H., Wahrer, M., Geyer, R., Frank, G., Gerardy-Schahn, R., Zahringer, U., and Schachner, M. (2001) Glycobiology 11, 373–384
28. Geyer, H., Baur, U., Liedtke, S., Schachner, M., and Geyer, R. (2001) Eur. J. Biochem. 268, 6587–6599
29. Ono, K., Nakayama, J., Angata, K., Reyes, L., Katsuyama, T., Arai, Y., and Fukuda, M. (1998) Glycobiology 8, 415–424
30. Close, B. E., and Colley, K. J. (1998) J. Biol. Chem. 273, 34586–34593
31. Kitamura-Kawaguchi, S., Kahata, S., and Arita, M. (2001) J. Biol. Chem. 276, 15696–15703
32. Kojima, N., Tachida, Y., and Tsuji, S. (1997) J. Biochem. (Tokyo) 122, 1265–1273
33. Muhlenhoff, M., Eckhardt, M., Bethe, A., Frosch, M., and Gerardy-Schahn, R. (1996) Curr. Biol. 6, 1188–1191
34. Muhlenhoff, M., Eckhardt, M., and Gerardy-Schahn, R. (1999) Curr. Opin. Struct. Biol. 8, 558–564
35. Muhlenhoff, M., Manegold, A., Windfuhr, M., Gotza, B., and Gerardy-Schahn, R. (2001) J. Biol. Chem. 276, 34066–34073
36. Nakayama, J., Angata, K., Ono, E., Katsuyama, T., and Fukuda, M. (1998) Pathol. Int. 48, 665–677
37. Seidenfaden, R., Gerardy-Schahn, R., and Hildebrandt, H. (2000) Eur. J. Cell Biol. 79, 680–688
38. Stoykova, L. I., Beesley, J. S., Grinspan, J. B., Glick, M. C. (2001) J. Neurosci. Res. 66, 497–505
39. Yang, P., Major, D., and Rutishauser, U. (1994) J. Biol. Chem. 269, 23039–23044