Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 1-6

THE GLUCOSE POLYMER OF PC12 CELLS IS SUSCEPTIBLE TO TRYPSINIZATION

Maija-Liisa Rasilo* and Tatsuya Yamagata

Laboratory of Glycoconjugate Research,
Mitsubishi Kasei Institute of Life Sciences,
Machida, Tokyo 194, Japan

Received January 3, 1989

SUMMARY: The glucose polymer of PC12 cells (Rasilo, M.-L. and Yamagata, T., 1988, FEBS Letters 227, 191-194 and Rasilo, M.-L. and Yamagata, T., 1988, Journal of Biochemistry, 104, 742-754) was found to be located on the cell surface. The polymer was liberated from the galactose-labeled cells with a trypsin treatment: maximally 65% of the glucose polymer was liberated, compared with 36% of the large glycopeptides. Even when the cells were incubated with the saline about one fourth of the polymer moved into the solution, but less than 8% of the large glycopeptides. Phosphatidyl inositol-specific phospholipase C failed to liberate the polymer.

Rat pheochromocytoma cells of line PC12 (1) have been found to serve well as a model system for the study of surface mediated cellular functions (2). In this connection glycoproteins, glycopeptides, glycosaminoglycans, and glycolipids of the cells have been the focus of active study (3-11). Recently we described the presence in PC12 cells of a glucose polymer composed almost solely of glucose and having a molecular weight of several millions (12, 13). The polymer was also found in neuronal cells of rat embryos but not in a neuroblastoma cell line studied (13). The location of this polymer has now been investigated by trypsinization of PC12 cells, using conditions comparable to those described in the literature (3, 14). Our present results suggest the localization of the glucose polymer on the surface of the cells.

MATERIALS AND METHODS

Cell Culture: PC12 cells (1), kindly provided by Dr. T. Amano of this institute, were cultured in RPMI 1640 medium (Nissui Pharmaceuti-

*Visiting Guest Researcher, Present address: Department of General Chemistry, Faculty of Agriculture and Forestry, University of Helsinki, Viikki, 00710 Helsinki, Finland.

Abbreviations used: PBS, phosphate buffered saline; PBS(+), PBS containing 100 mg/l each of $CaCl_2$ and $MgCl_2(6H_2O)$; Con A, concanavalin A; GP, glycopeptide; PIPLC, phosphatidylinositol specific phospholipase C.

0006-291X/89 $1.50

cal Co., Japan) containing 10% heat-inactivated (30 min, 56 °C) horse serum (Gibco, U.S.A.) and 5% pseudo fetal calf serum (precolostrum newborn calf serum, Mitsubishi Kasei Corporation, Japan) at 37 °C in 5% CO_2. The cells were grown on untreated tissue culture plastic dishes, surface area 75 cm^2 (Falcon Labware, U.S.A.), and passaged once every four days without treatment with trypsin.

Labeling of Cells, Treatment with Trypsin, PIPLC and Preparation of Pronase Digests: Cells in their late growth phase were labeled for 24 h with D-[1-^{3}H]galactose (Amersham, U.K., 6.9 Ci/mmol) at a concentration of 10 μCi/ml. The labeled cells were washed four times with 1 ml of 0.85% NaCl and treated with trypsin (Cooper Biomedical, 150 U/mg protein, 88% protein), 0.6 mg/ml in the saline (experiment I) or 0.05 mg/ml in PBS(+) (experiment II) for 30 min at 37 °C. Final volume was 1 ml in experiment I and 3 ml in experiment II. The incubation mixture was centrifuged with a low speed centrifuge (Kubota KN-70) at 700 rpm at room temperature. The supernatant, and the cell pellet suspended in the saline, were boiled for 10 min in a water bath. An equal amount of 0.2 M Tris-HCl buffer (pH 8.0) containing 20 mM $CaCl_2$ was added, and then 50 μl/ml of 1% pronase, Streptomyces griseus (Calbiochem, La Jolla, U.S.A., autodigested at 37 °C for 2 h): finally the system was incubated at 60 °C under toluene. New pronase (50 μl/ml of the suspension) was added twice a day and the incubation was continued for five days. The digests were boiled for 10 min, and then frozen and maintained at -20 °C until use.

In the case of PIPLC treatment, the labeled cells in a culture bottle were treated with 1 unit of the enzyme (100 units/mg protein, Kirin Breweries, Japan) in 2 ml of the saline for 60 min at 37 °C. The incubation mixture was centrifuged to obtain the supernatant and the cell pellet, both of which were treated as in the case of trypsinization experiments.

In control experiments the cells were incubated with 1 ml of the saline (experiment I), with 3 ml of PBS(+) (experiment II), and with 2 ml of the saline (for the PIPLC control) and processed like the trypsin treated cells. Viability of cells was measured by counting cells on a hematocytometer following staining with 0.1% trypan blue.

Gel Filtration and Affinity Chromatography: Gel filtration was carried out on a Bio-Gel P-30 column (1.0-1.2 x 90 cm) equilibrated in 0.2 M pyridine acetate (pH 5.0) containing 0.02% NaN_3. The void (Vo) and total volumes (Vt) of the columns were measured with Blue Dextran 2000 (Pharmacia, Sweden) and [1-^{3}H]galactose (Amersham, U.K.), respectively. Fractions of 1.1-1.2 ml were collected and aliquots of each fraction were analyzed for radioactivity. Fractions eluting in the void volume of the column were then analyzed on a Con A-Sepharose.

Affinity chromatography on Con A-Sepharose 4B (Pharmacia, Sweden) was carried out at room temperature on a column (1.0 x 15 cm) equilibrated in Con A-buffer (10 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.02% NaN_3). The preparation was dissolved in the Con A-buffer before loading onto the column. Glycopeptides were collected with Con A-buffer in twenty fractions of 1.2 ml each (Fraction Con A I). The column was then eluted with 10 mM methyl α-mannoside in Con A-buffer (20 fractions) to obtain fractions containing the glucose polymer (Fraction Con A II). Aliquots were measured for radioactivity.

Counting of Radioactivity: An aliquot of the sample solution was placed on a disk of glass filter paper (GF/C, Millipore Ltd., Japan), oven dried at 70 °C and counted for radioactivity in a vial containing a scintillation cocktail of 4 g 2,5-diphenyloxazole per liter of toluene. The counting was done using Beckman liquid scintillation counters LS-250, LS-1800, and LS-7800 or an LKB-Wallac 1214 RackBeta Exel.

RESULTS AND DISCUSSION

The glucose polymer is eluted in the void volume of a Bio-Gel P-30 column and retarded on a Con A-Sepharose column (12, 13). The behavior of the polymer following trypsinization was investigated after isolation in this way (Fig. 1). Table 1 shows that 19.1% and 36.4% of the large glycopeptides of cell surfaces (VoI+GP_{large}) were recovered in the supernatant after trypsin treatment in experiments I and II, respectively. But the recoveries of the glucose polymer (VoII) were 26.7% and 64.8% at the same time, showing that it is much more susceptible to trypsin than are the large glycopeptides. Since viability of the cells decreased by only 2% during incubation, liberation of the glucose

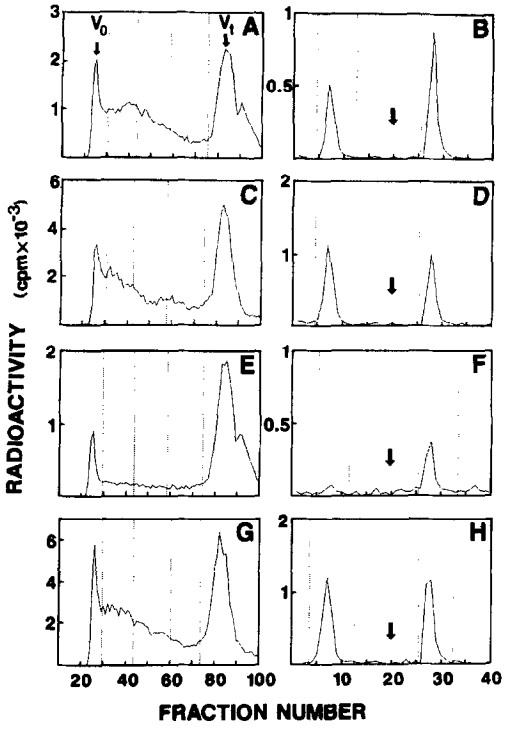

Fig. 1. Analysis of trypsinization of PC12 cells by gel filtration on Bio-Gel P-30 (panels A, C, E, G) and affinity chromatography on Con A-Sepharose (panels B, D, F, H). Panels A-D show results from trypsin treated, and panels E-H from control cells. Pronase digests of the supernatant from trypsin treated (0.6 mg/ml) (A) and control cells (E) were gel filtered on a column of Bio-Gel P-30. Relatively more of the glycopeptides, fractions 30-66 (A), was liberated with trypsin than in a control experiment (E). Fractions around the void volume, shown in panels A and E, were further fractionated on Con A-Sepharose, as shown in panels B and F, respectively. Radioactivity eluted with 10 mM methyl-α-mannoside represents the glucose polymer. Pronase digest on Bio-Gel P-30 obtained from trypsin treated cells is shown in panel C and that from control cells in G. Panels D and H show the fractionation on the Con A-Sepharose column of the voided fractions of panels C and G, respectively. Arrows indicate the start of elution with 10 mM methyl-α-mannoside.

TABLE 1: RELEASE OF THE GLUCOSE POLYMER BY TRYPSINIZATION§

	VoII*	VoI** + GP_{large}	GP_{medium}	GP_{short}	Sum
Experiment I					
Trypsinate Sup	77,930 **(26.7)**	225,376 **(19.1)**	149,747 (23.5)	84,678 (16.4)	537,731 (20.5)
Cells	213,723 **(73.3)**	953,698 **(80.9)**	488,641 (76.5)	432,031 (83.6)	2,088,093 (79.5)
Sum	291,653 (100)	1,179,074 (100)	638,388 (100)	516,709 (100)	2,625,824 (100)
Control Sup	35,048 **(14.0)**	40,831 **(3.9)**	27,366 (4.2)	27,470 (8.8)	130,715 (5.8)
Cells	215,046 **(86.0)**	1,013,211 **(96.1)**	617,579 (95.8)	283,005 (91.2)	2,128,841 (94.2)
Sum	250,094 (100)	1,054,042 (100)	644,945 (100)	310,475 (100)	2,259,556 (100)
Experiment II#					
Trypsinate Sup	202,690 **(64.8)**	273,174 **(36.4)**	36,089 (13.7)	17,088 (15.4)	529,041 (36.8)
Cells	109,946 **(35.2)**	476,962 **(63.6)**	226,781 (86.3)	93,548 (84.6)	907,237 (63.2)
Sum	312,636 (100)	750,136 (100)	262,870 (100)	110,636 (100)	1,436,278 (100)
Control Sup	82,945 **(28.4)**	32,800 **(8.4)**	3,174 (1.3)	3,495 (2.0)	122,414 (11.1)
Cells	208,882 **(71.6)**	355,756 **(91.6)**	239,833 (98.7)	170,273 (98.0)	974,744 (88.9)
Sum	291,827 (100)	388,556 (100)	243,007 (100)	173,768 (100)	1,097,158 (100)

§ Data are expressed in cpm. Percentage distributions in the various fractions are given in parentheses.

* VoII is the radioactivity eluting in the void volume of the Bio-Gel P-30 column (Vo) and retarded on the Con A-Sepharose column (Con A II), representing the glucose polymer.

** VoI is the radioactivity eluting in the void volume of the P-30 column (Vo) and excluded from the Con A Column (Con A I) and representing the large glycopeptides.

\# The supernatant fractions obtained after trypsinization in experiment II were fractionated on the P-30 column without pronase digestion.

polymer could not have occurred primarily from dead cells. Control experiments in the absence of trypsin indicated that the glucose polymer naturally tends to move into the supernatant: 14.0% and 28.4% of the glucose polymer (VoII) were recovered in the supernatant in the control experiments I and II, respectively. Only 3.9% and 8.4% of the large surface glycopeptides (VoI+GP_{large}) were found in the supernatant in the control incubations I and II, again indicating that the glucose polymer more easily detaches from the membranes. It is highly likely, therefore, that the glucose polymer liberated by trypsin is located on the outer surface of the cell membranes.

According to the data presented in Table 1, the glucose polymer was liberated from PC12 cells under conditions which liberated 17.5% (experiment I) and 22.7% (experiment II) of the label incorporated into glycoproteins. The conditions are comparable to those used by Margolis et al., which resulted in the liberation of 10% of glycoproteins from the cell surface of PC12 cells (3). Possibly our values are higher because the caluculation method we used did not take account of the

fraction eluting at the total volume of the column, which may correspond to the soluble fraction of Margolis et al. (3). The more efficient liberation of radioactivity in experiment II than in experiment I is likely due to the use of Ca^{2+} in an incubation medium in experiment II. Ca^{2+} is known to increase the activity of trypsin (14).

NILE (NGF inducible long external) glycoprotein of PC12 cells is also trypsin sensitive (15), suggesting its localization on the cell surface. This was later confirmed by staining it at the cell surface with its specific antibody (16).

The glucose polymer was not found to have the free reducing saccharide at the reducing terminal in earlier work (13), suggesting to us the presence of an aglycan part. Now, our finding that at least half of the glucose polymer is susceptible to trypsin treatment strongly suggests that the aglycan part is a peptide.

PIPLC has proved to be a useful tool to liberate glycoproteins anchored to phosphatidylinositol on cell membranes (17, 18). In our experiments, the large and medium sized glycopeptides were enriched in the supernatant by PIPLC treatment (Table 2), showing them to originate from glycoproteins anchored to glycosylated phosphatidylinositol. This agrees with the earlier finding that in PC12 cells there are four proteins anchored to the cell membrane via phosphatidylinositol linkages (19). By contrast, the amount of glucose polymer liberated from the labeled PC12 cells was almost the same in the presence of PIPLC (20.6%) as in the absence of PIPLC (21.9%, Table 2). Although these results suggest that the glucose polymer is not attached to the cell

TABLE 2: THE GLUCOSE POLYMER UNAFFECTED BY PIPLC TREATMENT§

	VoII*	VoI** + GP_{large}	GP_{medium}	GP_{short}	Sum
PIPLC Sup	51,187 **(20.6)**	335,118 **(21.3)**	96,420 (13.1)	51,481 (9.5)	534,209 (19.4)
Cells	197,838 **(79.4)**	901,869 **(78.7)**	636,816 (86.9)	488,130 (80.5)	2,224,653 (80.6)
Sum	249,025 (100)	1,236,987 (100)	733,236 (100)	539,611 (100)	2,758,862 (100)
Control Sup	43,290 **(21.9)**	73,234 **(4.7)**	44,520 (6.0)	29,051 (6.1)	190,095 (6.4)
Cells	154,347 **(78.1)**	1,477,517 **(95.3)**	698,064 (94.0)	447,690 (95.9)	2,777,618 (93.6)
Sum	197,637 (100)	1,550,751 (100)	742,584 (100)	476,741 (100)	2,967,713 (100)

§ Data are expressed in cpm. Percentage distributions in the various fractions are given in parentheses.

* VoII is the radioactivity eluting in the void volume of the Bio-Gel P-30 column (Vo) and retarded on the Con A sepharose column (Con A II), representing the glucose polymer.

** VoI is the radioactivity eluting in the void volume of the P-30 column (Vo) and excluded from the Con A column (Con A I) and representing the large glycopeptides.

membrane via phosphatidylinositol linkages, PIPLC does not necessarily cleave all phosphatidylinositol-anchored glycoproteins (18), and the possibility that the glucose polymer is anchored in this way is not fully ruled out.

ACKNOWLEDGMENTS: We are indebted to Dr. T. Amano for the supply of pheochromocytoma cells of PC12 and for stimulating discussion throughout this work, and to Drs. H. Ikezawa and R. Taguchi for their help in performing the experiments using PIPLC. We are grateful to Mses. Yoshiko Fujimoto and Izumi Hosokane for their valuable technical assistance. This work was in part supported by The Finnish Cultural Foundation and The Magnus Ehrnrooth Foundation.

REFERENCES

1. Greene, L.A., and Tischler, A.S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2424-2428.
2. Greene, L.A., Tischler, A.S. (1982) In Advances in Cellular Neurobiology (Federoff, S. and Herz, L., Eds.), Vol. III, pp. 373-414. Academic Press, New York.
3. Margolis, R.K., Salton, S.R.J., and Margolis, R.U. (1983) J. Biol. Chem. 258, 4110-4117.
4. Margolis, R.K., Salton, S.R.J., and Margolis, R.U. (1983) J. Neurochemistry 41, 1635-1640.
5. Margolis, R.K., Greene, L.A., and Margolis, R.U. (1986) Biochemistry 25, 3453-3468.
6. Salton, S.R.J., Shelanski, M.L., and Greene, L.A. (1983) J. Neurosci. 3, 2420-2430.
7. Richter-Landsberg, C., Lee, V.M., Salton, S.R.J., Shelanski, M.L., and Greene, L.A. (1984) J. Neurochem. 43, 841-848.
8. Sajovic, P., Moraru, E., Greene, L.A., and Shelanski, M.L. (1984) J. Neurosci. 6, 82-93.
9. Schwarting, G.A., Gajewski, A., Babero, A., Tischler, A.S., and Costopoulos, D. (1986) Neuroscience 19, 647-656.
10. Ariga, T., Macala, L.J., Saito, M., Margolis, R.K., Greene, L.A., Margolis, R.U., and Yu, R.K. (1988) Biochemistry 27, 52-58.
11. Shimamura, M., Hayase, T., Ito, M., Rasilo, M.-L., and Yamagata, T. (1988) J. Biol. Chem. 263, 12124-12128.
12. Rasilo, M.-L., and Yamagata, T. (1988) FEBS Letters, 227, 191-194.
13. Rasilo, M.-L., and Yamagata, T. (1988) J. Biochem. 104, 742-754.
14. Laskowski, M. (1955) in Methods in Enzymology, (Colowick, S. P., and Kaplan, N. O., Eds.), 2, 26-36. Academic Press, New York.
15. McGuire, J.C., Greene, L.A., and Furano, A.V. (1978) Cell 15, 357-365.
16. Salton, S.R.J., Richter-Landsberg, C., Greene, L.A., and Shelsnski, M.L. (1983) J. Neurosci. 3, 441-454.
17. Ikezawa, H., and Taguchi, R. (1981) in Methods in Enzymology, (Lowenstein, J. M., Ed.), 71, 731-741. Academic Press, New York.
18. Low, M.G. (1987) Biochem. J. 244, 1-13.
19. Margolis, R.K., Goossen, B., and Margolis, R.U. (1988) Biochemistry 27, 3454-3458.