Phosphatidylinositol-Anchored Glycoproteins of PC12 Pheochromocytoma Cells and Brain†

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ABSTRACT: PC12 pheochromocytoma cells and cultures of early postnatal rat cerebellum were labeled with [^{3}H]glucosamine, [^{3}H]fucose, [^{3}H]leucine, [^{3}H]ethanolamine, or sodium [^{35}S]sulfate and treated with a phosphatidylinositol-specific phospholipase C. Enzyme treatment of [^{3}H]glucosamine- or [^{3}H]fucose-labeled PC12 cells led to a 15-fold increase in released glycoproteins. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, most of the released material migrated as a broad band with an apparent molecular size of 32 000 daltons (Da), which was specifically immunoprecipitated by a monoclonal antibody to the Thy-1 glycoprotein. A second glycoprotein, with an apparent molecular size of 158 000 Da, was also released. After treatment with endo-β-galactosidase, 40–45% of the [^{3}H]glucosamine or [^{3}H]fucose radioactivity in the phospholipase-released glycoproteins was converted to products of disaccharide size, and the molecular size of the 158-kDa glycoprotein decreased to 145 kDa, demonstrating that it contains fucosylated poly(*N*-acetyllactosaminyl) oligosaccharides. The phospholipase also released labeled Thy-1 and the 158-kDa glycoprotein from PC12 cells cultured in the presence of [^{3}H]ethanolamine, which specifically labels this component of the phosphatidylinositol membrane-anchoring sequence, while in the lipid-free protein residue of cells not treated with phospholipase, Thy-1 and a doublet at 46/48 kDa were the only labeled proteins. At least eight early postnatal rat brain glycoproteins also appear to be anchored to the membrane by phosphatidylinositol. Sulfated glycoproteins of 155, 132/134, 61, and 21 kDa are the predominant species released by phospholipase, which does not affect a major 44-kDa protein seen in [^{3}H]ethanolamine-labeled brain cultures. The 44–48- and 155/158-kDa proteins may be common to both PC12 cells and brain.

Most integral membrane proteins are anchored by a balance of interactions between relatively hydrophobic and polar polypeptide domains with the hydrophobic core of the lipid bilayer and the surrounding medium, respectively. However, it has recently been recognized that an extremely diverse group of proteins is anchored to the plasma membrane through a covalently attached glycosylphosphatidylinositol moiety. These include various enzymes (alkaline phosphatase, 5′-nucleotidase, acetylcholinesterase, alkaline phosphodiesterase I, trehalase, and the p63 proteinase of *Leishmania major*), a number of lymphocyte antigens including the Thy-1 glycoprotein, decay accelerating factor (a complement regulatory protein), the variant surface glycoprotein of *Trypanosoma brucei*, the smallest [120 kilodalton (kDa)[1]] component of the neural cell adhesion molecule, and a rat liver heparan sulfate proteoglycan [for a review, see Low (1987)].

On the basis of those proteins for which detailed information is available, the anchoring structure generally consists of a phosphatidylinositol molecule, whose 1,2-diacylglycerol moiety is embedded in the membrane bilayer and is responsible for anchoring, and an oligosaccharide of variable structure and composition. The oligosaccharide is linked to the membrane phosphatidylinositol via a glycosidic linkage with a glucosamine that has a free amino group and via a nonreducing terminal mannose 6-phosphate to the hydroxyl of an ethanolamine residue, which is amide linked via its amino group to the α-carboxyl of the C-terminal amino acid.

In an attempt to evaluate the extent to which this novel membrane-anchoring mechanism is employed in nervous tissue, we have used biosynthetic labeling with [^{3}H]ethanolamine and a phosphatidylinositol-specific phospholipase C to study the prevalence of this structure in cultures of early postnatal rat cerebellum and in a homogeneous population of rat PC12 pheochromocytoma cells, whose complex carbohydrates have previously been studied in some detail (Margolis et al., 1983a,b, 1986, 1987; Salton et al., 1983a,b). PC12 cells grown in serum-containing medium display many of the neurotransmitter properties and morphological features of normal adrenal chromaffin cells. In the presence of nanogram levels of nerve growth factor, they cease to divide, extend long microtubule-containing processes, and acquire electrical excitability, increased sensitivity to acetylcholine, and other properties of sympathetic neurons [for a review, see Greene and Tischler (1982)]. We present evidence that Thy-1 and three other PC12 cell proteins employ this same membrane-anchoring mechanism but otherwise differ from each other in several respects, while at least eight phosphatidylinositol-anchored glycoproteins are present in brain.

Experimental Procedures

Cell Culture Conditions. Rat PC12 pheochromocytoma cells were routinely cultured on collagen as described by Greene and Tischler (1976) in RPMI 1640 medium (GIBCO, Grand Island, NY) containing 10% heat-inactivated (30 min, 56 °C) horse serum and 5% fetal calf serum (both from

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[1] Abbreviations: PIPLC, phosphatidylinositol-specific phospholipase C; NGF, nerve growth factor; N-CAM, neural cell adhesion molecule; SDS, sodium dodecyl sulfate; Da, dalton(s); HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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Hazleton Research Products, Denver, PA). They were labeled for 48 h with [6-^{3}H]glucosamine (50 μCi/mL, 29 Ci/mmol), [5,6-^{3}H]fucose (10 μCi/mL, 56 Ci/mmol), or [4,5-^{3}H]leucine (45 μCi/mL, 60 Ci/mmol), all obtained from New England Nuclear, and for 18 h with [1-^{3}H]ethanolamine (30 μCi/mL, 30 Ci/mmol; Amersham). PC12 cells and brain cultures were labeled for 24–48 h with sodium [^{35}S]sulfate (100–200 μCi/mL) in Eagle's basal diploid medium (GIBCO no. 420–1300) containing 0.2 mM sodium sulfate.

Reaggregate cultures of 1–3-day postnatal rat cerebellum were prepared and grown for 2–3 days in plastic tissue culture dishes essentially as described by Trenkner and Sidman (1977) and labeled for 18–48 h with [^{3}H]glucosamine, [^{3}H]-ethanolamine, and sodium [^{35}S]sulfate using the isotope concentrations given above for PC12 cells.

Isolation of Proteins Released by Phosphatidylinositol-Specific Phospholipase C. Phosphatidylinositol-specific phospholipase C (PIPLC) purified from culture supernatants of *Bacillus thuringiensis* was generously provided by Dr. Martin Low (Columbia University). (One microliter of this preparation hydrolyzed 0.3 μmol of [^{3}H]phosphatidylinositol per minute, when incubated in pH 7 HEPES buffer containing 0.1% deoxycholate.) Monolayer cultures of PC12 cells were gently rinsed with 25 mM HEPES-buffered saline (pH 7.35) and then treated for 1 h at 37 °C with a 1:400 dilution of PIPLC in HEPES-buffered saline. Equivalent control cells were treated under the same conditions but without addition of enzyme. Brain cultures were detached from the culture dishes by addition of HEPES buffer and treated with PIPLC in a volume of 0.8 mL. The PIPLC incubation medium (which will be referred to as "release medium") was centrifuged initially for 5 min at 400 rpm and then for 1 h at 140000g to ensure complete sedimentation of small cell debris.

For some experiments, PC12 cells were also cultured for 10 days with 50 ng/mL 2.5S nerve growth factor in RPMI medium with 1% horse serum (βNGF; obtained from the Technology Transfer Office of the Research Foundation of the State University of New York, Albany, NY), and the release medium was centrifuged for 20 min at 10000*g* followed by 1 h at 140000*g*.

After addition of unlabeled glucosamine, fucose, leucine, ethanolamine, or sodium sulfate carrier to a concentration of 5 mM, the release medium was concentrated on a Centricon-10 filter (Amicon) and exchanged several times with saline to remove free precursor radioactivity. The cells were removed from the culture dish by repeated forceful aspiration, and aliquots of the resulting suspension were used for protein assay (Lowry et al., 1951) and for SDS–PAGE in comparison with the PIPLC-released glycoproteins. [^{3}H]Ethanolamine-labeled cells were also extracted with 0.75 mL of chloroform–methanol (1:1 v/v) to obtain a "lipid-free protein residue" which was used for electrophoresis.

Treatment with Endo-β-Galactosidase. [^{3}H]Glucosamine- and [^{3}H]fucose-labeled release media were incubated for 1 h at 37 °C in 0.1 mL of 50 mM sodium acetate buffer, pH 6, in the presence or absence of 4 milliunits of endo-β-galactosidase from *Escherichia freundii* (Miles Laboratories). After incubation, the volume was made to 1 mL with 0.2 M NaCl, and the digestion products were analyzed by gel filtration on Sephadex G-50 (Centricon-10 filtrate) and by SDS–PAGE (Centricon-10 retentate).

Immunoprecipitations. Release medium from [^{3}H]-glucosamine-labeled PC12 cells were precleared by mixing for 1 h with 10 mg of preswollen protein A–Sepharose beads (Pharmacia) in 50 mM phosphate-buffered saline, pH 7.2, and

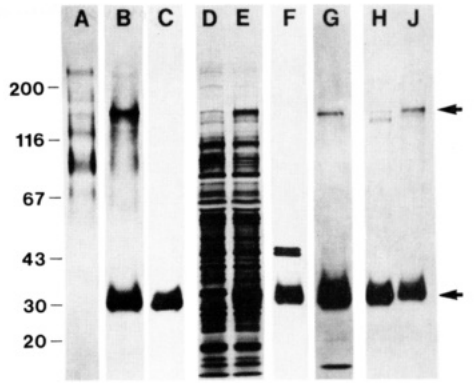

FIGURE 1: Fluorograph of 7.5–15% SDS–polyacrylamide slab gel showing PC12 cell proteins released by phosphatidylinositol-specific phospholipase C (PIPLC) or labeled by [^{3}H]ethanolamine in the lipid-free protein residue of PC12 cells. Release media of [^{3}H]-glucosamine-labeled PC12 cells incubated for 1 h in the absence (lane A) or presence (lane B) or PIPLC and immunoprecipitate of PIPLC-released glycoproteins with monoclonal antibody to Thy-1 (lane C); release media of [^{3}H]leucine-labeled PC12 incubated for 1 h in the absence (lane D) or presence (lane E) of PIPLC; lipid-free protein residue of [^{3}H]ethanolamine-labeled PC12 cells after extraction with chloroform–methanol (lane F); proteins in the release medium of [^{3}H]ethanolamine-labeled cells treated with PIPLC (lane G); glycoproteins released from [^{3}H]fucose-labeled PC12 cells after (lane H) and before (lane J) treatment with endo-β-galactosidase. Numbers at the left indicate the positions of molecular weight standards (given as $M_r \times 10^{-3}$), and arrows at the right indicate the positions of the 158-kDa glycoprotein and Thy-1.

then incubated for 18 h at 4 °C with 10 μL of anti-Thy-1.1 ascites fluid (MRC OX-7 clone; obtained from Bioproducts for Science, Indianapolis, IN). This solution was added to 10 mg of new protein A–Sepharose beads and mixed for 1 h at room temperature. The beads were then washed several times with PBS, and the bound proteins were eluted by heating the beads in sample buffer prior to SDS–PAGE.

Release media from [^{3}H]glucosamine-, [^{3}H]leucine-, and [^{35}S]sulfate-labeled PC12 cells or brain cultures were similarly treated with 10 μL of a rabbit antiserum to rat/mouse N-CAM, which was kindly provided by Dr. Urs Rutishauser (Case Western Reserve University).

Electrophoresis. Cells and released proteins were heated for 5 min at 100 °C in sample buffer containing sodium dodecyl sulfate (SDS) and electrophoresed on 7.5–15% SDS–polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970), followed by staining with Coomassie Blue and fluorography.

Results and Discussion

PC12 Cell Glycoproteins Released by Phosphatidylinositol-Specific Phospholipase C. Treatment of PC12 cells labeled with [^{3}H]glucosamine or [^{3}H]fucose with phosphatidylinositol-specific phospholipase C (PIPLC) resulted in a 15-fold increase in the release of labeled glycoproteins, expressed as cpm per milligram of cell protein and determined after removal of free precursor radioactivity by Centricon-10 ultrafiltration. SDS–PAGE of the released glycoproteins revealed the presence of two major components with apparent molecular sizes of 32 000 and 158 000 Da, whereas they were not seen among those glycoproteins released (in much smaller amounts) in the absence of phospholipase (Figure 1A,B,J).

To determine whether there were other proteins released by PIPLC which were not significantly labeled with glucosamine or fucose, we also evaluated its effects on [^{3}H]-leucine-labeled cells. Over 40 [^{3}H]leucine-labeled proteins could be distinguished by SDS–PAGE in the media of cells incubated in either the absence or presence of PIPLC (Figure

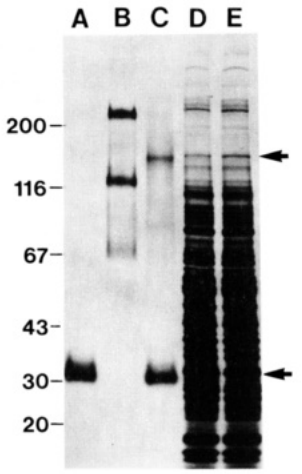

FIGURE 2: Fluorograph comparing proteins released by PIPLC from NGF-treated (lanes A, B, D, and E) and untreated PC12 cells (lane C). Release media obtained from [^{3}H]glucosamine-labeled cells in the presence (lanes A and C) and absence (lane B) of PIPLC and from [^{3}H]leucine-labeled cells in the presence (lane D) and absence (lane E) of PIPLC. Note that the 158-kDa glycoprotein released by PIPLC (arrow) is much less prominent in media from NGF-treated PC12 cells (lanes A and D) as compared to PC12 cells not treated with NGF (lane C). Other details are as in the legend to Figure 1.

1D,E). Only the bands at 32 000 and 158 000 Da were increased in intensity in the medium from phospholipase-treated cells, and the large majority of [^{3}H]leucine-labeled proteins which were not affected by PIPLC accounts for the fact that the enzyme did not produce any significant overall increase in the release of labeled PC12 cell proteins.

In nerve growth factor treated PC12 cells (Figure 2), PIPLC produced less or no increase in the release of the 158-kDa glycoprotein (labeled with either [^{3}H]leucine or [^{3}H]glucosamine), indicating that its concentration may be decreased by nerve growth factor. PIPLC treatment produced no significant increase in the release of [^{35}S]sulfate-labeled proteins, and neither the 32 000-Da nor the 158 000-Da glycoproteins, whose release was stimulated by PIPLC, incorporated [^{35}S]sulfate radioactivity.

Immunoprecipitations with Anti-Thy-1 and Anti-N-CAM. Thy-1 is known to possess a phosphatidylinositol membrane protein anchor (Low & Kincade, 1985; Tse et al., 1985; Fatemi et al., 1987) and was assumed to account for the major PIPLC-release glycoprotein. However, Thy-1 usually migrates on SDS–PAGE with an apparent molecular size of approximately 25 000 Da, although the brain glycoprotein has a molecular weight of 17 500 determined by sedimentation equilibrium measurements, and both the brain and thymocyte forms have identical polypeptides of M_r 12 500 to which are attached three asparagine-linked oligosaccharides of different structures (Parekh et al., 1987). In view of the somewhat higher apparent molecular size of the major PIPLC-released glycoprotein, which consistently migrated on SDS–PAGE as a broad band immediately above a 30 000-Da carbonic anhydrase standard, we confirmed its identity as Thy-1 by demonstrating that it was immunoprecipitated by the OX-7 monoclonal antibody to rat Thy-1 (Figure 1C). The finding that the Thy-1 glycoprotein in PC12 cells is anchored to the membrane via phosphatidylinositol is noteworthy in view of the earlier report that after short-term extraction of PC12 cell monolayer cultures with Triton X-100, the Thy-1 glycoprotein remains associated with the detergent-resistant cytoskeletal fraction (Richter-Landsberg et al., 1985).

It has also been reported that the smallest (120 kDa) component of the neural cell adhesion molecule N-CAM is anchored to the membrane by phosphatidylinositol (He et al., 1986; Sadoul et al., 1986; Hemperly et al., 1986), although this component does not appear to be present in PC12 cells, which contain primarily the 140-kDa and (in much smaller amounts) the 180-kDa components (Stallcup & Beasley, 1985). No radioactivity was immunoprecipitated from the PIPLC-released PC12 cell glycoproteins by an antiserum to rat/mouse N-CAM, although a single band migrating at 120 kDa was immunoprecipitated from the PIPLC-released proteins of rat brain microsomes (biosynthetically labeled with sodium [^{35}S]sulfate administered by intracerebral injection to 30-day-old rats; data not shown).

Ethanolamine Labeling Studies. PC12 cells were also cultured in the presence of [^{3}H]ethanolamine to specifically label this component of the phosphatidylinositol membrane protein anchor (Medof et al., 1986; Fatemi et al., 1987; Jemmerson & Low, 1987; Howard et al., 1987). Approximately 95% of the incorporated radioactivity was in the form of chloroform–methanol-extractable lipids, which migrated ahead of the tracking dye front on SDS–PAGE of whole cells dissolved in sample buffer. When the lipid-free protein residue of [^{3}H]ethanolamine-labeled cells was used for electrophoresis, fluorography revealed a major labeled protein with the mobility of Thy-1 and two additional proteins with apparent molecular sizes of 46 000 and 48 000 Da (Figure 1F). The 158 000-Da glycoprotein previously identified as being released together with Thy-1 by PIPLC was not seen on fluorographs of the lipid-free protein residue of [^{3}H]ethanolamine-labeled cells, presumably because it is soluble in the chloroform–methanol used for lipid extraction. The solubility in chloroform–methanol of some phosphatidylinositol-anchored glycoproteins of PC12 cells and brain (see below) is not surprising but has not, to our knowledge, been previously reported. The 158-kDa glycoprotein apparently contains ethanolamine in its PIPLC-susceptible membrane anchor, since both it and Thy-1 were released into the medium after PIPLC treatment of [^{3}H]-ethanolamine-labeled PC12 cells (Figure 1G). The 46- and 48-kDa proteins seen in fluorographs of the lipid-free protein residue of [^{3}H]ethanolamine-labeled cells, but not in the medium after treatment of the cells with PIPLC, presumably represent two phosphatidylinositol-anchored proteins which are resistant to hydrolysis by PIPLC both when used under our usual conditions and when a 4-fold higher enzyme concentration was used. [For a discussion of other phosphatidylinositol-anchored proteins which are resistant to release by PIPLC, see Low (1987).] Only the larger of these two PIPLC-resistant components was also labeled by [^{3}H]fucose and [^{3}H]glucosamine.

The specificity of ethanolamine labeling for this component of the phosphatidylinositol membrane protein anchor has previously been demonstrated in other studies (Medof et al., 1986; Fatemi et al., 1987; Jemmerson & Low, 1987; Howard et al., 1987) and is supported by our finding that this precursor labeled only four PC12 proteins, two of which are released by PIPLC. The small number of [^{3}H]ethanolamine-labeled proteins also argues against the possibility of any significant "recycling" of ethanolamine radioactivity into other molecules under our labeling conditions.

Effects of Endo-β-Galactosidase on Phosphatidylinositol-Anchored Glycoproteins of PC12 Cells. We have previously reported the presence of poly(*N*-acetyllactosaminyl) oligosaccharides in a number of PC12 cell glycoproteins, some of which (such as the NILE glycoprotein) are regulated by nerve growth factor (Margolis et al., 1986). Moreover, in agreement with our demonstration that these oligosaccharides

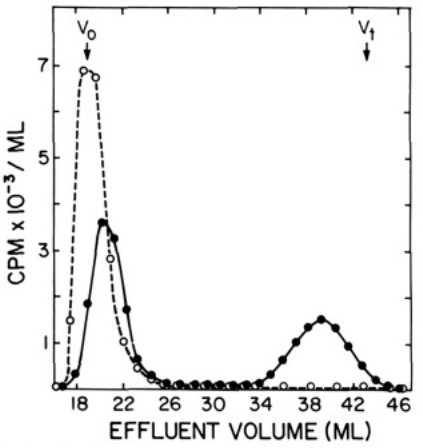

FIGURE 3: Sephadex G-50 elution pattern of glycoproteins released by PIPLC from [^{3}H]glucosamine-labeled PC12 cells before (○) and after (●) treatment with endo-β-galactosidase. The column (0.9 × 65 cm) was eluted with 0.2 M NaCl containing 0.1% deoxycholate. Equal amounts of radioactivity were applied for the two samples, and after endo-β-galactosidase treatment, 40% of the radioactivity eluted between 33 and 45 mL, in the position expected for disaccharides.

are generally not present in postnatal brain glycoproteins, it has been reported that the thymocyte but not the brain form of Thy-1 contains poly(*N*-acetyllactosaminyl) oligosaccharides (Parekh et al., 1987). In view of these findings, we examined the susceptibility of phosphatidylinositol-anchored glycoproteins of PC12 cells to endo-β-galactosidase, which specifically degrades oligosaccharides containing poly(*N*-acetyllactosaminyl) units.

Endo-β-galactosidase treatment of glycoproteins released by PIPLC from [^{3}H]glucosamine- or [^{3}H]fucose-labeled PC12 cells resulted in the conversion of 40–45% of the labeled material into products of approximately disaccharide size (Figure 3), as compared to 20–25% of the radioactivity in glycoproteins released in the absence of PIPLC. SDS–PAGE of the PIPLC-released glycoproteins after treatment with endo-β-galactosidase demonstrated that the 158-kDa glycoprotein decreased in intensity and a new band appeared with an apparent molecular size of 145 kDa, whereas there was only a slight increase in the mobility of Thy-1 (Figure 1H,J). The latter finding is in agreement with the results of our previous studies, in which there was very little labeling in the region of Thy-1 after SDS–PAGE of PC12 cells whose poly(*N*-acetyllactosaminyl) oligosaccharides in cell-surface glycoproteins were specifically labeled with [^{14}C]galactose (Margolis et al., 1986). Since a gel filtration pattern essentially identical with that shown in Figure 3 was obtained after endo-β-galactosidase digestion of the PIPLC-released glycoproteins from [^{3}H]fucose-labeled PC12 cells, it can be concluded that the poly(*N*-acetyllactosaminyl) oligosaccharides in the 158-kDa glycoprotein are fucosylated.

Phosphatidylinositol-Anchored Glycoproteins of Brain. In [^{3}H]glucosamine-labeled cultures of 2-day postnatal rat cerebellum, the proteins released by PIPLC had apparent molecular sizes of 155, 132/134, 94, 61, 51, 21, and 19 kDa (Figure 4A,B). The doublet at 132/134 kDa often appears only as a broad band after longer exposures of the fluorograph and in [^{3}H]ethanolamine-labeled cultures (see below) but can be clearly seen as two distinct components (together with the highly labeled 61-kDa glycoprotein) in shorter exposures, which do not reveal the minor glycoproteins released by PIPLC (Figure 4E). When electrophoresed next to PIPLC-released glycoproteins of PC12 cells, it is also clear that the 155-kDa brain glycoprotein migrates slightly more rapidly than the 158-kDa glycoprotein of PC12 cells (Figure 4F).

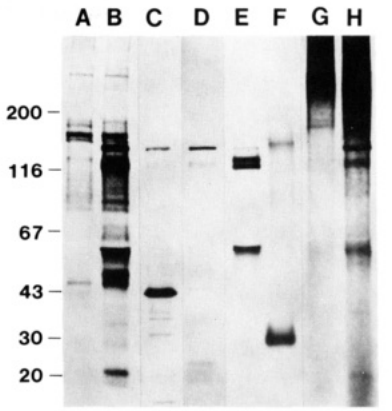

FIGURE 4: Fluorograph of PIPLC-released and [^{3}H]ethanolamine-labeled proteins of early postnatal rat cerebellar cultures. (Lanes A and B) Release media from [^{3}H]glucosamine-labeled cultures incubated in the absence and presence of PIPLC, respectively; (lane C) lipid-free protein residue of [^{3}H]ethanolamine-labeled culture; (lane D) proteins released by PIPLC from [^{3}H]ethanolamine-labeled culture; (lane E) short-exposure fluorograph of [^{3}H]glucosamine-labeled brain glycoproteins released by PIPLC, showing 132/134-kDa doublet, and comparison with [^{3}H]glucosamine-labeled PC12 cell glycoproteins released by PIPLC (lane F); (lanes G and H) release media from [^{35}S]sulfate-labeled cultures incubated in the absence and presence of PIPLC, respectively.

The major [^{3}H]glucosamine-labeled glycoproteins released by PIPLC (155, 132/134, 61, and 21 kDa) were clearly apparent in [^{35}S]sulfate-labeled brain cultures, demonstrating that these are sulfated glycoproteins (Figure 4G,H). PIPLC also increased the intensity of labeling at the top of the gel where the chondroitin sulfate and heparan sulfate proteoglycans of brain are known to migrate, suggesting that one or both of these may have been released by the phospholipase. However, this question will require further study since there is a significant release of these proteoglycans even in the absence of enzyme.

In [^{3}H]ethanolamine-labeled brain cultures, most of the labeling in the lipid-free protein residue is present in a 44-kDa protein, accompanied by a major band at 155 kDa and six minor components with apparent molecular sizes of 132/134, 37, 35, 30.5, and 16.7 kDa (Figure 4C). Only the 155-kDa and (to a much lesser extent) the 132/134-kDa glycoproteins were released to a significant degree by PIPLC (Figure 4D). The major 44-kDa component present in the lipid-free protein residue of [^{3}H]ethanolamine-labeled cultures is not apparent in the PIPLC release medium of [^{3}H]glucosamine-labeled brain cultures, a property it shares with the 46/48-kDa [^{3}H]ethanolamine-labeled proteins of PC12 cells, as described above. Conversely, the less prominent [^{3}H]glucosamine-labeled proteins in the PIPLC release medium either are apparently not efficiently labeled by [^{3}H]ethanolamine or are extracted by chloroform–methanol. The larger number of phosphatidylinositol-anchored proteins in brain cultures is consistent with their greater cellular heterogeneity as compared to PC12 cells, although 44–48- and 155/158-kDa phosphatidylinositol-anchored proteins may be common to both PC12 cells and brain. The slightly smaller molecular size of the 155/158-kDa glycoprotein in brain cultures may be attributable to the absence of poly(*N*-acetyllactosaminyl) oligosaccharides in most brain glycoproteins (Margolis et al., 1986), as supported by the finding that the PIPLC-released glycoproteins of [^{3}H]glucosamine-labeled brain cultures were not affected by treatment with endo-β-galactosidase.

Thy-1 labeling (or release by PIPLC) was not apparent in these cultures, in agreement with other reports that little or no Thy-1 is present during early brain development (Morris,

1985; Bolin & Rouse, 1986), nor was N-CAM evident as a labeled 120-kDa [^{3}H]glucosamine-, [^{3}H]ethanolamine-, or [^{35}S]sulfate-labeled protein which was released by PIPLC. However, a small amount of material migrating at 120 kDa could be immunoprecipitated with anti-N-CAM from the release media of [^{35}S]sulfate-labeled brain cultures. As mentioned above, we found that a 120-kDa anti-N-CAM-immunoprecipitable protein was released from [^{35}S]sulfate-labeled microsomes of mature brain, and the 120-kDa component of N-CAM has also been reported to be released by a different PIPLC from cultured rat C6 glioma cells (He et al., 1986). These results suggest that the 120-kDa component of N-CAM is not expressed to a significant degree in our early postnatal brain cultures.

Conclusions. We have found that in a homogeneous PC12 cell population there are at least four proteins which appear to be anchored to the plasma membrane by a glycosylphosphatidylinositol linkage. The major component is Thy-1. This is accompanied by two proteins having apparent molecular sizes of 46 and 48 kDa and which are not released by PIPLC, although they are biosynthetically labeled with [^{3}H]ethanolamine, and by a PIPLC-released 158-kDa glycoprotein which is soluble in chloroform–methanol and contains fucosylated poly(*N*-acetyllactosaminyl) oligosaccharides. None of these latter three bands can be identified with previously investigated proteins having known functional roles, and the biological significance of Thy-1 in nervous tissue also remains unclear. In comparison with PC12 cells, we found a significantly greater number of phosphatidylinositol-anchored glycoproteins in cultures of early postnatal rat cerebellum, including five major sulfated glycoproteins.

In most cases, the biosynthetic incorporation of labeled sugar precursors does not necessarily indicate the presence of N- or O-glycosidic oligosaccharides linked directly to the protein moiety, since these sugars have frequently also been identified as components of the phosphatidylinositol membrane anchor structure (e.g., glucosamine, mannose, galactose, and galactosamine). Therefore, except in special cases such as the identification of poly(*N*-acetyllactosaminyl) oligosaccharides in specific phosphatidylinositol-anchored proteins, their classification as conventional *glyco*proteins must remain tentative pending structural characterization of their oligosaccharides.

The physiological functions of the phosphatidylinositol membrane protein anchor are likely to be different (when functionally relevant at all) among many of the highly diverse proteins which employ this linkage mechanism. However, it is reasonable to assume that a glycosylphosphatidylinositol anchor would confer a greater degree of lateral mobility within the outer leaflet of the membrane bilayer and would also facilitate the selective release and/or uptake of certain enzymes, cell adhesion molecules, or other proteins having a role in cell-surface events. The generation at the cell surface by specific phospholipases C of 1,2-diacylglycerol (an activator of protein kinase C) and of glycosylinositol phosphates also suggests that these products may serve as second messengers, which could be delivered to their intracellular target sites by diffusion across the lipid bilayer or by receptor-mediated endocytosis, respectively. The presence of at least four phosphatidylinositol-anchored proteins on the plasma membrane of a single cell type suggests that this novel linkage mechanism is probably widely employed to serve a number of biological functions in nervous and other tissues.

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