IDENTIFICATION OF PHOSPHOGLYCOPROTEINS OBTAINED FROM RAT BRAIN

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1. Introduction

Nerve endings and synaptosomal plasma membranes have been reported to contain a high concentration of glycoproteins [1,2] and more recently it has been shown that myelin and synaptic membranes contain Concanavalin A receptors [3]. Glycopeptides that possess concanavalin A receptor activities have been recovered from brain tissue by proteolytic treatment of defatted tissue and affinity chromatography utilizing Concanavalin A-Sepharose [4,5]; mannose rich glycopeptides that bind strongly to Concanavalin A are especially enriched in the myelin-rich fraction and are present in synaptic plasma membranes.

Since recent attention has focused on cyclic 3′,5′-adenosine monophosphate (cAMP) dependent phosphorylation of membrane proteins [6,7], we sought to determine whether some of these phosphate-containing, membrane-bound proteins were glycoproteins. Several phosphoproteins are known to be glycoproteins. Rhodopsin [8–11] is a glycoprotein that contains a hexasaccharide; the molecule is phosphorylated by ATP or GTP in the presence of Mg^{2+}. Phosphorylation is not stimulated by cyclic nucleotides. Phosvitin, the phosphoprotein of hen's egg yolk contains 6.5% carbohydrate [12]. The heteropolysaccharide moiety isolated from phosvitin contained mannose, galactose, *N*-acetylglucosamine and *N*-acetylneuraminic acid [13]. Phosphate residues were shown to be linked to serine in the polypeptide chain [12,14]. Phosphoserine-containing glycoproteins have been obtained from bone [15] and teeth [16].

In this communication, we describe a phosphoglycoprotein that contains a phosphate group which is attached to the heteropolysaccharidic side-chain.

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2. Materials and methods

Male albino rats (Holtzman, Madison, Wis.) were injected twice at 24 h intervals with 1 mCi $Na_2{}^{32}PO_4$ (carrier-free, in 1 ml isotonic saline) intraperitoneally. Animals were sacrificed 24 h after the last injection. Glycoproteins that contain mannose-rich heteropolysaccharide chains were isolated from the defatted brain tissue as described by Susz et al. [17]. Briefly, brain was extracted with chloroform–methanol (2:1 and 1:2, v/v) to remove lipids. The defatted residue was dissolved in 5% sodium dodecyl sulfate (SDS) and this detergent was exchanged for sodium deoxycholate by dialysis. Affinity chromatography on Con A-Sepharose 4B was carried out in the presence of 1% sodium deoxycholate. Procedures for polyacrylamide gel electrophoresis and gel filtration of the labeled product eluted from Con A-Sepharose are described in legends to figs.1 and 2. Radioactivity on polyacrylamide gels was determined by solubilizing 2 mm sections cut from the slab in hydrogen peroxide and counted by liquid scintillation in aquasol fluid.

The phosphorylated glycopeptides were isolated from the labeled phosphoglycoprotein starting with brain tissue or glycoproteins recovered from the Con A Sepharose column. Rats were injected with [^{32}P] PO_4 as before and sacrificed 24 h after the last

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injection. Pooled brain tissue was homogenized in cold 10% trichloroacetic acid. The method of Greenberg and Rothstein [18] was used to free the insoluble material of acid soluble P, lipid P, and nucleic acids. The trichloroacetic acid insoluble material (up to 10 extractions with 5% trichloroacetic acid) was further extracted with chloroform–methanol (2:1, 1:2, v/v) to remove residual lipids. Excess trichloroacetic acid was removed by extraction with ether. Glycopeptides were prepared from the dried residue by treatment with papain [5]. Glycosaminoglycans and the remaining nucleic acids were removed from the soluble material obtained by centrifugation by precipitation with cetyl pyridinium chloride [1]. The glycopeptides were freed of small molecules by exhaustive ultrafiltration on UM-2 membranes using Amicon (Lexington, Mass.) ultrafiltration cells, and by gel filtration on Bio-Gel P-2 columns (5 × 30 cm.). The mannose-rich glycopeptides were recovered from the glycopeptide preparation by adsorption on Con A-Sepharose 4B [4]. After their elution with 2% α-methylmannoside, the material was desalted by ultrafiltration and gel filtration. The mannose-rich glycopeptides were applied to a Dowex 1-X8 (chloride) column. Neutral mannose-rich glycopeptides were washed from the column with water, and the bound glycopeptides were eluted with 1 M NaCl. The glycopeptides were then desalted desalted by ultrafiltration and gel filtration.

In order to determine whether the phosphoglyco-

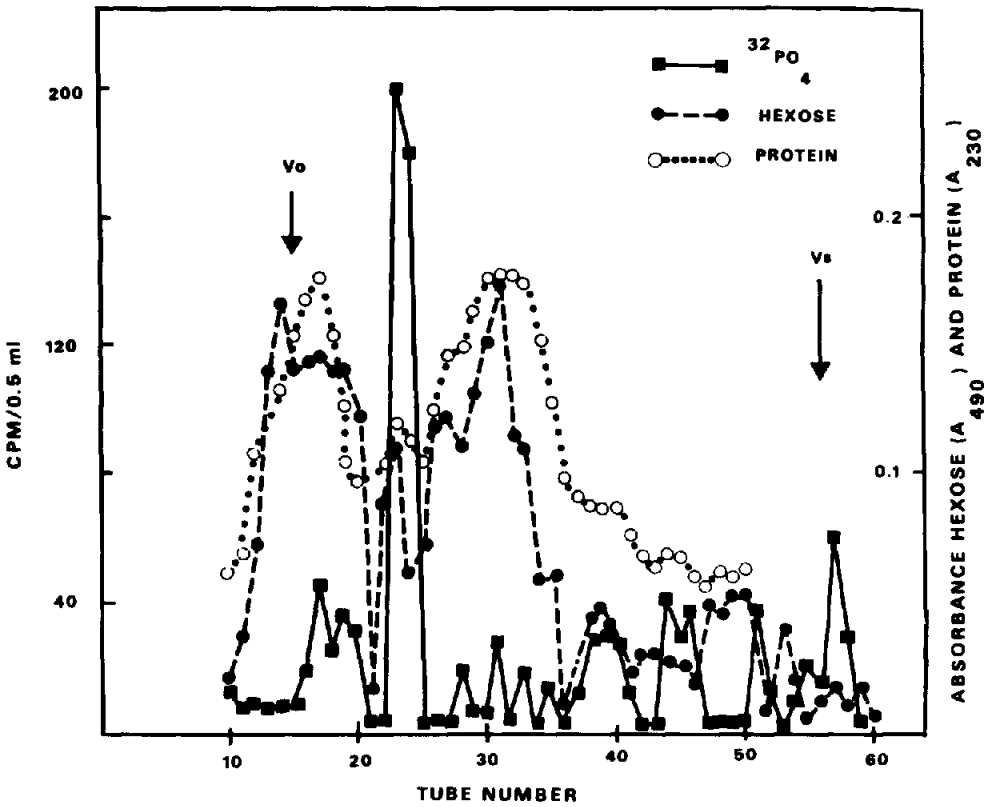

Fig.1. Gel filtration of [^{32}P]phosphate labeled, Con A-binding glycoproteins that were recovered from a Con A-Sepharose column. Sephadex G-50 (fine) columns (1.5 × 90 cm) were pre-equilibrated with 2% sodium dodecyl sulfate. 150 drops (approx. 5 ml) were collected per tube. The position of the void volume (dextran blue) and the elution of salts (chromate) are indicated by the arrows. Radioactivity was determined by counting (Nuclear Chicago Scintillation Counter) of 0.5 ml aliquots from each fraction after the addition of 20 ml of Aquasol. Hexose was determined by the phenol–sulfuric acid method [22] using 0.1 ml aliquots. Protein was estimated by determining absorbancy at 230 nm.

protein serves as a substrate in the membrane-phosphorylation system, the procedure of Johnson et al. [6] was used. Synaptosomal fractions from brain were recovered by the procedure of Johnson et al. [19] and incubated with and without added cyclic AMP in the presence of γ-[^{32}P]ATP [6]. After the incubation, the Con A-binding glycoproteins were isolated from the incubation medium as described above by stopping the reaction by heating and the addition of 1% SDS.

3. Results

Approx. 4% of the total SDS-solubilized protein from whole brain was found to bind to Concanavalin A-Sepharose, and were eluted with 2% α-methyl-mannoside in 1% sodium deoxycholate. Eight major and 4 to 6 minor periodic acid–Schiff positive bands, each of which corresponds to protein bands that stained with Coomassie blue, were detected by polyacrylamide gel electrophoresis of the Con A-binding fraction. The major radioactive peak migrated to a position corresponding to an apparent mol. wt. of 60 000 (standard, bovine serum albumin). The Con A-binding glycoproteins were fractionated on Sephadex G-50 (1.5 X 90 cm) columns that had been equilibrated with 2% SDS. A phosphate-labeled glycoprotein fraction was obtained (fig.1). Upon SDS-electrophoresis, this fraction was shown to contain a major and two minor bands (fig.2).

The mannose-rich, Con A-binding glycopeptides that were obtained by proteolytic treatment of defatted whole brain tissue (or from the Con A-binding glycoprotein fraction) were fractionated by anion exchange chromatography. Approximately 80–88% of the glycopeptide–carbohydrate applied to Dowex 1-X8 (chloride) was washed from the column with water. Approximately 12 to 20% of the carbohydrate, and all of the ^{32}P radioactivity was adsorbed to the column and eluted with 1 M sodium chloride. The molecular weight of the labeled glycopeptide was estimated to be 2200 (gel filtration on calibrated Bio-Gel P-4 columns). These acidic glycopeptides did not contain *N*-acetylneuraminic acid or sulfate; the absence of ribose was established by g.l.c.

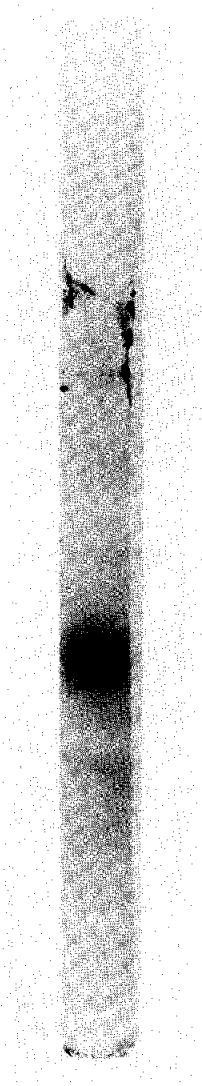

Fig.2. Discontinuous polyacrylamide gel electrophoresis of the phosphoglycoprotein fraction obtained by gel filtration chromatography on G-50 Sephadex (fig.1.). Approx. 100 μg protein in 100 μl was applied to the gel. A discontinuous buffer system [17] was used; the gel system consisted of three layers, equal in amount, with acrylamide gel concentrations of 4%, 7.5%, and 10%. Electrophoresis was performed at room temperature at a constant 2 mA. Proteins were stained in a 0.25% Coomassie stain in methanol/acetic acid/water (5/1/4, v/v/v) overnight and destained by diffusion in the solvent and 10% acetic acid. The major stained band was cut out, dissolved in hydrogen peroxide, and was shown to contain radioactive phosphorus by counting. Radioactivity in the minor bands only slightly exceeded that of the background and identification of these bands as phosphoglycoproteins is uncertain. The origin is at the top of the figure.

analysis [5]. The material was devoid of absorbancy in ultraviolet light above 230 nm. N.m.r. studies [20] revealed the absence of phosphoserine, phospholipid, and diester phosphate linkages. None of the label could be extracted into an organic phase. Treatment of the phosphorylated glycopeptides with leucine aminopeptidase did not release radioactive phosphate, although amino acids were released by the enzyme. Treatment of the phosphorylated glycopeptides with α-mannosidase released mannose, but phosphorylated hexose was not liberated. In this regard, Colonna and Lampen [21] have reported that phosphomannose residues are not suitable substrates for α-mannosidase.

The radioactive, Con A-binding glycopeptides were hydrolyzed in 0.5 N HCl for 18 h at 100°C, and the hydrolyzate was applied to a column of Dowex 50 (hydrogen). All of the radioactivity and hexose of the original material was washed from the column with water. All of the glucosamine was recovered from the column by elution with 3 N HCl. The hexose and radioactivity that failed to bind to Dowex 50 was applied to columns of Dowex 1-X8 (chloride). Approx. 66% of the applied hexose was washed from the column with water; elution with 1 M NaCl provided a recovery of the remaining 34% of the applied hexose and all of the radioactivity. The ratio of hexose to glucosamine was 3:1, and this data suggests that one of the three hexose units is phosphorylated.

Synaptosomal fractions that had been incubated in the presence and absence of added cAMP and γ-[^{32}P]ATP were extracted with SDS and the extracts were applied to columns of Con A-Sepharose. No radioactivity was found in the glycoproteins that bound to Con A; this was true if the incubation was carried out in the presence or absence of added cAMP. However, radiophosphorus was found in the fraction that did not bind to Con A-Sepharose, indicating that phosphorylation was active, as reported [6]. Also, it was found that the phosphorylated mannose-rich, Con A-binding glycopeptides isolated after incubation were not radioactive. It was also established that the Con A-binding neutral mannose-rich glycopeptides did not serve as substrates in the in vitro phosphorylating system, with and without added cAMP, of Johnson, et al. [6]. It is concluded that the conditions of the in vitro phosphorylating system of Johnson et al. [6] are not capable of supporting the phosphorylation of the phosphoglycoprotein studied here.

4. Discussion

The phosphorylated, Con A-binding, mannose rich glycopeptides appear to be the only phosphate-containing heteropolysaccharide chains associated with brain glycoproteins [20]. Structural, metabolic, subcellular and anatomic distribution studies pertaining to these phosphoglycoproteins is under investigation. In view of the occurrence of glycoproteins that contain phosphoserine residues in their polypeptide chains, it is suggested that the phosphorylated protein studied here be called phosphoglycoproteins, in order to distinguish it from the glycophosphoproteins such as phosvitin.

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