

# Isolation of Phosphooligosaccharide/Phosphoinositol Glycan from Caveolae and Cytosol of Insulin-stimulated Cells

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**Abstract.** A phosphooligosaccharide has been proposed as a second messenger of insulin. It is believed to be structurally related to the carbohydrate moiety of phosphatidylinositol glycan anchors of many cell surface proteins. Herein we demonstrate that [<sup>32</sup>P]phosphate in freshly isolated adipocytes and [<sup>3</sup>H]galactose in cultured hepatoma cells (H4IIE) labeled the same set of three different glycolipids. With all three, the radiolabel was made water soluble by phosphatidylinositol(glycan)-specific phospholipase C or D catalyzed hydrolysis. We isolated the three phospholipase C-released substances. One of them was susceptible to nitrous acid deamination, indicative of a hexosamine with a free amino group. This phosphooligosaccharide structure had an apparent molecular mass between tetra- and pentaglycose by gel filtration. By anion-exchange chromatography it was separated into two differently charged and interconvertible species. Adi-

pocytes stimulated with insulin accumulated the nitrous acid sensitive phosphooligosaccharide: after stimulation the intracellular level of free phosphooligosaccharide increased threefold within 5 min, fell off during the next few minutes and then remained at a slightly elevated level. After insulin stimulation the intracellular concentration of free phosphooligosaccharide was >1,000-fold higher than in the incubation medium. When prepared from rat livers on a preparative scale, the oligosaccharide was also found to exhibit insulinomimetic effects on protein phosphorylation of insulin target proteins in intact adipocytes. After subcellular fractionation of adipocytes the lipid-bound [<sup>32</sup>P]phosphooligosaccharide of the plasma membrane was found to be localized in plasma membrane domains apparently corresponding to caveolae. Lipid-bound [<sup>32</sup>P]phosphooligosaccharide was found also in the microsomal fraction.

**T**HE mechanisms for the acute effects of insulin action remain incompletely known in spite of detailed characterization of its cellular receptor, established target proteins' reversible phosphorylation in response to the hormone and identification of a large number of insulin activated protein kinases. Recently a pathway for growth factor control of nuclear events was described (Egan and Weinberg, 1993). Insulin may use the same pathway for some of its long-term effects, e.g., stimulation of mitogenesis (Törnkvist et al., 1994; White and Kahn, 1994), and parts thereof for its short-term effects.

A few years ago the concept of second messengers in insulin action was revived in the form of a phosphorylated oligosaccharide or phosphoinositol glycan (Jarett et al., 1981; Larner et al., 1981; Kelly et al., 1986; Saltiel and Cuatrecasas, 1986; Kelly et al., 1987a,b; Mato et al., 1987a). In support of this concept we demonstrated that a crude preparation of the phosphooligosaccharide faithfully mimicked the effects of insulin on protein phosphorylation and dephosphorylation in intact rat adipocytes (Alemany et

al., 1987), but insulin's enhancement of glucose transport was not affected by the oligosaccharide (Kelly et al., 1987b). The precursor of the phosphooligosaccharide is believed to be a glycolipid, a phosphatidylinositol glycan, which upon hydrolysis by phospholipase C gives rise to the phosphooligosaccharide and diacylglycerol. With this in mind we demonstrated that specific diacylglycerols could mimic the effect of insulin to stimulate glucose transport in adipocytes, thus suggesting a unifying model of insulin signal transduction in short-term metabolic control, involving the dual mediators phosphooligosaccharide and diacylglycerol (Strålfors, 1988).

A feature of the phosphooligosaccharide is its structural kinship to a number of well-characterized phosphatidylinositol glycan anchors that attach many proteins to the plasma membrane (Thomas et al., 1990). The phosphooligosaccharide is thus believed to contain inositolphosphate, hexosamine with a free amino group and a number of monosaccharide residues (Saltiel and Cuatrecasas, 1986; Mato et al., 1987a,b; Merida et al., 1988; Macaulay and Larkins, 1990; Gaulton, 1991; Suzuki et al., 1991; Gottschalk, 1992).

The lack of structural data, which are lacking because the oligosaccharide has not yet yielded to attempts to pu-

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rify it, has delayed significant progress over the last years. Direct determination of chemical constituents have been done on crude and ill defined preparations only. Metabolic labeling experiments have been challenged as irreproducible in recent investigations (Deeg et al., 1992; Whatmore et al., 1993). Here we demonstrate the isolation of radiolabeled phosphooligosaccharide that has insulin effects on target protein phosphorylation in adipocytes. The phosphooligosaccharide is associated with caveolae-rich fractions of the plasma membrane, it is released in a phospholipase catalyzed reaction, and it rapidly accumulates in adipocytes when they are stimulated by insulin.

## Materials and Methods

### Materials

[<sup>32</sup>P]Phosphate and D-[<sup>3</sup>H]galactose were obtained from Amersham International (Amersham, UK), Sprague Dawley rats from Alab (Stockholm, Sweden), and H4IIE hepatoma cells from American Type Culture Collection (Rockville, MD). All solvents used were of pro analysis quality or better and were supplied by Riedel de Haen (Seelze, Germany) or Merck (Darmstadt, Germany). Porcine insulin was from Novo Nordisk Biolabs (Copenhagen, Denmark). Phosphatidylinositol phospholipase C from *Bacillus cereus* and glycosyl-phosphatidylinositol phospholipase D from bovine serum were from Boehringer Mannheim GmbH (Mannheim, Germany). Mouse anti-caveolin monoclonal antibodies was from Chemicon Intl. Inc. (Temecula, CA). Polyclonal rabbit anti-glucose transporter-4 and anti-glucose transporter-1 antibodies were from Calbiochem-Novabiochem (La Jolla, CA), and polyclonal rat anti-Na,K-ATPase alpha 2 subunit fusion protein (Shyjan and Levenson, 1989) from Upstate Biotechnology, Inc. (Lake Placid, NY). Suppliers of other materials and equipment are indicated below.

### Isolation and Incubation of Adipocytes

Adipocytes were prepared from the epididymal fat pads of Sprague Dawley rats (120–180 g) by collagenase digestion (Strålfors and Honnor, 1989). Cells (final concentration 100  $\mu$ l packed cell volume per ml) were freshly incubated in Krebs-Ringer buffer (0.13 M NaCl, 5 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>) containing 20 mM Hepes, pH 7.40, 3.5% (wt/vol) defatted bovine serum albumin, 100 nM phenylisopropyladenosine, 0.5 U/ml adenosine deaminase and 2 mM D-glucose, at 37°C on a shaking waterbath. Cells were labeled with [<sup>32</sup>P]phosphate (1 mCi/ml) for 2 h (Strålfors and Honnor, 1989).

Incubations with [<sup>32</sup>P]phosphate were terminated by removing the radioactive medium and washing the cells three times in incubation medium without radiolabel. Insulin at 1 nM concentration was then added (and in parallel a control incubation without insulin) and the cells were incubated for the indicated time periods. Incubation medium was collected by centrifugation and immediately frozen. The packed cells were dispersed in methanol and frozen.

### Hepatoma Cell Culture and Incubation

Rat hepatoma cells H4IIE were grown in DMEM medium (GIBCO BRL, Gaithersburg, MD) containing 5.5 mM glucose, 10% fetal calf serum, 50 UI/ml penicillin, and 50  $\mu$ g/ml streptomycin in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. At confluence cells were labeled by incubating with D-[<sup>3</sup>H]galactose for 18 h. Incubations were terminated by removing the incubation medium and washing three times with medium lacking the radiolabel, removing the medium, and adding methanol. The H4IIE cells were responsive to insulin as evidenced by stimulation of glycogen synthesis and of DNA synthesis three- to fourfold by the hormone (Törnkvist et al., 1994).

### Extraction of Cellular Water-soluble Substances and Lipids

Cellular lipids were extracted with CH<sub>3</sub>OH/CHCl<sub>3</sub>/0.1 M KCl/HCl (1:2:1:0.01 by vol). The aqueous phase (cytosolic fraction) was collected and analyzed as described below. 1 vol CHCl<sub>3</sub> and silicic acid (preactivated by

heating at 110°C) was then added to the lipid-containing organic phase. After 30 min the solvent was decanted off and the silicic acid washed twice with CHCl<sub>3</sub>/CH<sub>3</sub>OH/HCl (6:1:0.04 by vol) and then twice with the same solvent mixture excluding the HCl. Polar lipids were eluted from the silicic acid using four extractions with CH<sub>3</sub>OH at 37°C (Mato et al., 1987a). The eluted material was evaporated to dryness under vacuum.

The aqueous phase, containing the water-soluble substances, was dried down under vacuum and fractionated by gel filtration (Biogel P2, 1.6  $\times$  50 cm; Biorad Labs., Richmond, CA) in 0.5% (vol by vol) CH<sub>3</sub>COOH. The eluate between 1.8  $\times$  v<sub>0</sub> and 2.2  $\times$  v<sub>0</sub> (approximately the elution positions of hexaglutose and triglutose, respectively) was collected and subjected to HPLC partition chromatography on GlycoPak N (below).

### Separation of Polar Lipids by Thin-Layer Chromatography

The extract of polar lipids was applied to a silicic acid plate (10  $\times$  10 cm, HPTLC; Merck, St. Louis, MO) and developed in CHCl<sub>3</sub>/CH<sub>3</sub>OH/Acetone/CH<sub>3</sub>COOH/H<sub>2</sub>O (10:2:4:2:1 by vol). The indicated area was then scraped off the dried plate and lipids extracted from the silicic acid with methanol as above. This lipid fraction was applied to a second thin-layer plate developed in CHCl<sub>3</sub>/CH<sub>3</sub>OH/NH<sub>4</sub>OH/H<sub>2</sub>O (45:45:3.5:10 by vol) (Mato et al., 1987a). The indicated region was scraped off the plate and this lipid fraction was extracted from the silicic acid as above.

### Hydrolysis with Phosphatidylinositol(Glycan)-specific Phospholipase C or D

The lipids obtained from the thin-layer chromatographies were suspended in a small volume of 25 mM Tris-borate buffer, pH 7.5, or sodium phosphate buffer, pH 6.8. The pH was determined and, if necessary, adjusted with 1 M Tris-base. Phosphatidylinositol-specific phospholipase C from *B. cereus* or glycosyl-phosphatidylinositol phospholipase D from bovine serum, respectively, was added and the mixture incubated for 3 h at 37°C. At the end of the incubation period 1 vol of CHCl<sub>3</sub>/CH<sub>3</sub>OH/HCl (2:1:0.005) was added and the mixture vortexed. The aqueous phase was collected and dried under vacuum.

### HPLC Partition-Chromatography, Gel Filtration, and Ion-Exchange Chromatography

A polymeric HPLC packing, developed for partition-chromatography separation of oligosaccharides (GlycoPak N; Millipore Corp., Bedford, MA), was operated isocratically at 0.3 ml per min with CH<sub>3</sub>CN/H<sub>2</sub>O (65:35 by vol) as the mobile phase. Samples were dissolved in 0.2 ml CH<sub>3</sub>CN/H<sub>2</sub>O (50:50 by vol) and fractions of 0.6 ml were collected. The breakthrough volume corresponded to fraction 7.

For analytical gel filtration of the radiolabeled substances Biogel P2 (1.0  $\times$  30 cm; Biorad Labs.) in 50 mM CH<sub>3</sub>COONH<sub>4</sub>, pH 6.0, was used at 0.1 ml per min and 0.4-ml fractions were collected. Samples were evaporated to dryness and dissolved in the equilibration buffer. The gel filtration column was calibrated with a partial hydrolyzate of Dextran T500 (Pharmacia LKB Nuclear, Gaithersburg, MD): 0.1 g Dextran in 1 ml 0.1 M HCl for 4 h at 100°C, followed by lyophilization.

Anion-exchange chromatography was performed at pH 6.0 on MA7P+-column (4.6  $\times$  30 mm; Biorad Labs.) equilibrated in 5 mM CH<sub>3</sub>COONH<sub>4</sub> at 0.5 ml/min. Samples were evaporated to dryness and applied in equilibration buffer and eluted with a linear gradient to 1 M CH<sub>3</sub>COONH<sub>4</sub> over 20 min.

### Subcellular Fractionation and Immunoblotting

Adipocytes were prelabeled with [<sup>32</sup>P]phosphate and homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.25 M sucrose (with protease inhibitors 10  $\mu$ M leupeptin, 1  $\mu$ M pepstatin, 1  $\mu$ M aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) using a motor-driven teflon/glass homogenizer at room temperature. All subsequent procedures were carried out at 0–4°C. A plasma membrane-containing fraction was obtained by centrifugation at 16,000 g for 20 min (Oka and Czech, 1984). The pellet was resuspended in 25 mM Hepes, pH 7.1, 0.15 M NaCl. Half of this plasma membrane fraction was treated with 1% (wt/vol) Triton X-100 till the solution cleared and solid sucrose was added to 40% (wt/vol). The solution was transferred to a centrifuge tube and layers of 25 and 0% sucrose were successively layered on top of it. After centrifugation at 200,000 g (average) for 6.5 h (TLS-55 rotor; Beckman Instrs., Fullerton, CA), the material

floating at the 25–0% interface was collected and referred to as the caveolae-rich fraction (Sargiacomo et al., 1993; Chang et al., 1994). The microsomal fraction was pelleted by centrifugation of the 16,000-g supernatant at 160,000 g (average) for 60 min (SW41 rotor; Beckman). Highly purified plasma membranes were isolated by sucrose density gradient centrifugation of the 16,000-g membranes (McKeel and Jarett, 1970; Karnieli et al., 1981) and a caveolae-rich fraction was isolated from this plasma membrane preparation as above.

After SDS-PAGE (10% acrylamide) separated proteins were electrophoretically transferred to a polyvinylidene difluoride blotting membrane (Immobilone-P; Millipore) in 25 mM Tris, 190 mM glycine, 20% (vol/vol) methanol, 0.05% (wt/vol) sodium dodecyl sulfate, at 50 mA for 20 h. The membrane was rinsed with water and stored dry until probing with antibody. The wetted membrane was saturated with gelatin, dry milk, or bovine serum albumin with 0.1% (wt/vol) Tween-20. After this blocking procedure the membrane was incubated with anti-caveolin monoclonal antibodies, anti-glucose transporter-4 antibodies, anti-Na,K-ATPase alpha 2 subunit antibodies, or anti-glucose transporter-1 antibodies in the same solution for 1 h. Bound antibodies were then detected with horseradish peroxidase conjugated anti-rabbit IgG, adhering to the ECL protocol and reagents from Amersham.

### Miscellaneous Procedures

For large scale preparation of phosphooligosaccharide, rat livers (300 g) from 120–180-g male rats were homogenized in 0.9% NaCl and a membrane fraction isolated by successive centrifugations at 5,000 g for 5 min and 30,000 g for 3 h. The high-speed membrane pellet was extracted and lipids fractionated in the same way as described above for radiolabeled cells (Mato et al., 1987a).

For analysis of protein phosphorylation, adipocytes were isolated and prelabeled with [<sup>32</sup>P]phosphate as described above. Substances were examined for effects on protein phosphorylation by addition of adipocyte suspension (70 μl packed cell volume per ml final concentration) to tubes with the substances to be tested and incubation for 30 min at 37°C on a shaking water-bath as described (Alemany et al., 1987). Total cell proteins were then prepared for SDS-PAGE as described (Strålfors and Honnor, 1989).

For nitrous acid deamination, samples were dried down under vacuum and redissolved in 40 μl 50 mM sodium acetate, pH 4.0, containing 0.25 M sodium nitrite and incubated at room-temperature (ca 23°C) for 3 h (Ferguson, 1988). Incubations were terminated by drying under vacuum and the immediate application to HPLC partition-chromatography (above).

Radioactivity determinations were done by liquid scintillation counting (Ready-Gel; Beckman) or for <sup>32</sup>P, in experiments with sufficient radioactivity, by its cerenkov-radiation. <sup>32</sup>P-labeling patterns on thin-layer chromatography plates or dried SDS-PAGE gels were visualized by radioimaging (Fujix Bas 1000 bioimaging analyzer; Fuji) or autoradiography with Hyperfilm β-max (Amersham), respectively.

Protein determination was carried out according to Bradford (1976).

## Results

### Isolation of [<sup>32</sup>P]Phosphooligosaccharide from [<sup>32</sup>P]Phosphate-labeled Adipocytes

Freshly isolated rat adipocytes were incubated with [<sup>32</sup>P]phosphate (Fig. 1) and a lipid extract (see Materials and Methods) of the cells was applied to thin-layer chromatography in an acidic mobile phase (Fig. 2 a). Radiolabeled material in the indicated area ( $2.2 \times 10^4$  cpm) was taken to a second thin-layer chromatographic separation in a basic mobile phase (Fig. 2 b). The material in the indicated area ( $7 \times 10^3$  cpm) was subjected to hydrolysis by phosphatidylinositol-specific phospholipase C from *B. cereus* and partitioned between CHCl<sub>3</sub> and water-CH<sub>3</sub>OH. The material that partitioned into the water-phase was analyzed by thin-layer chromatography in the same basic solvent system (Fig. 2 c): The band labeled 5 represents part of the unhydrolyzed polar-lipid material that partly partitions into the water-phase and band 1 is material that has bound “irreversibly” at the sample application. Bands 2, 3 (bands

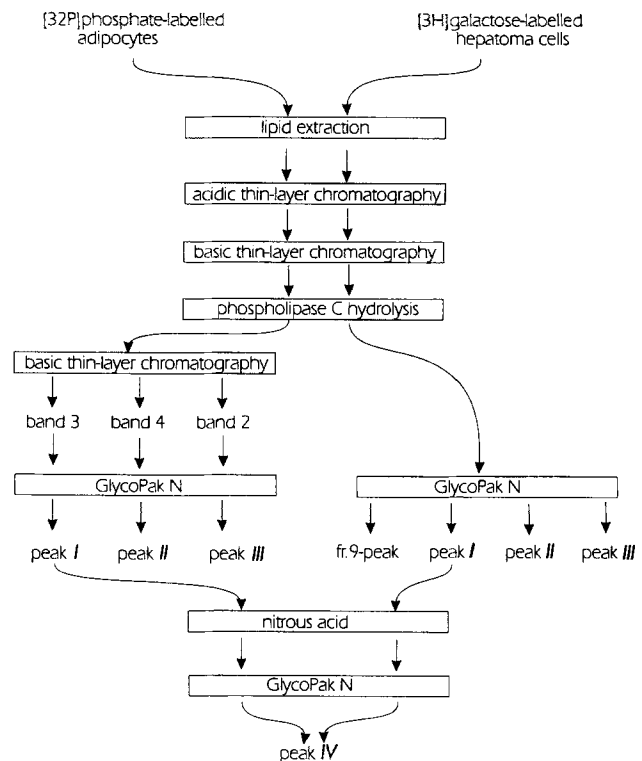


Figure 1. Outline of isolation and identification of phosphooligosaccharide.

2 + 3:  $1 \times 10^3$  cpm), and 4 ( $1 \times 10^3$  cpm) represent material that has been produced by the phospholipase catalyzed hydrolysis (and correspond to 30% of the labeled lipid material). Bands 2, 3, and 4 partitioned virtually completely into the water-phase, since they were not detectable in the organic-phase (not shown). Phosphatidylinositol is also a substrate for this lipase, but we verified that it was removed by the combined thin-layer chromatographies. All

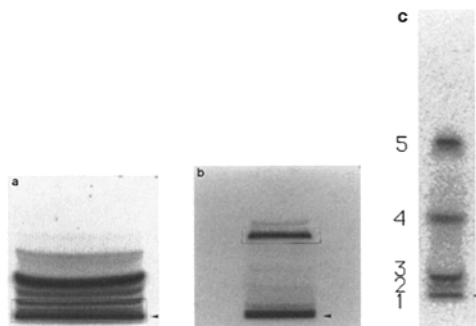


Figure 2. Autoradiograph of thin-layer chromatography of lipid extract from <sup>32</sup>P-labeled adipocytes. Isolated adipocytes were incubated with [<sup>32</sup>P]phosphate and lipids were extracted as described in Materials and Methods. (a) First thin-layer chromatography in acidic mobile phase. The indicated area was scraped off the plate and lipids extracted with methanol for (b), second thin-layer chromatography in basic mobile phase. The indicated area was scraped off the plate and subjected to treatment with phosphatidylinositol-glycan-specific phospholipase C. (c) Polar, water-soluble products were extracted by two-phase partitioning (Materials and Methods) and rechromatographed in the basic mobile phase. Numbers indicate the different radiolabeled substances that were detected, arrow indicates application origin.

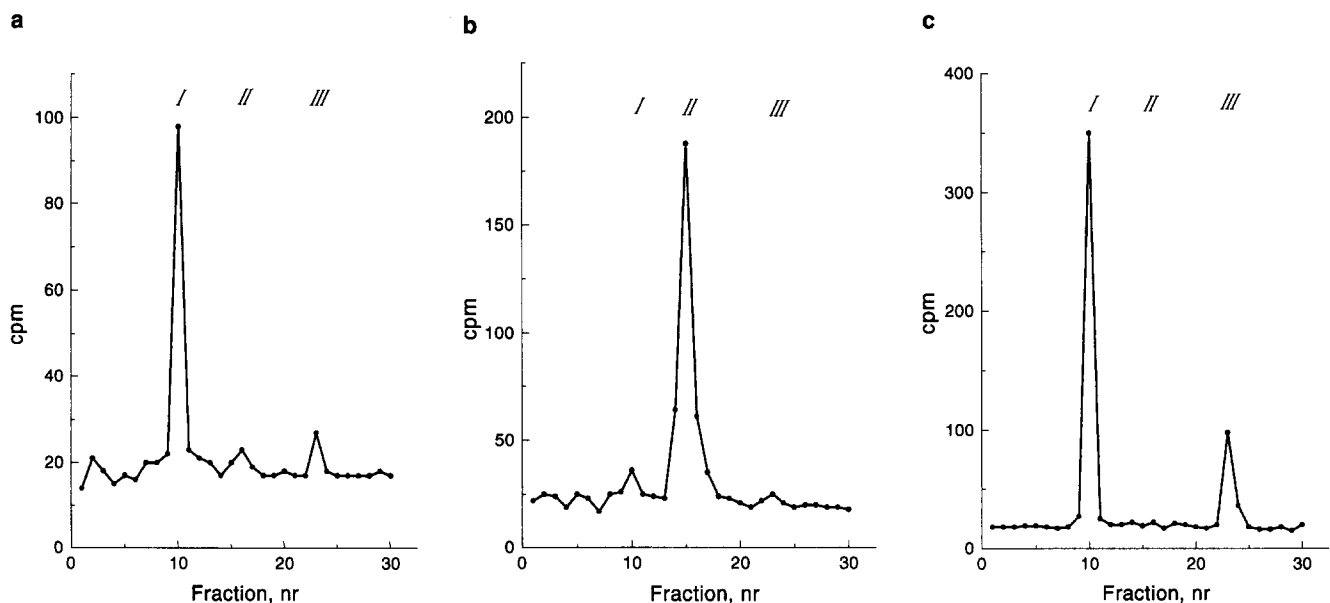
three bands (2, 3, and 4) in Fig. 2 *c* were also generated by phosphatidylinositol-glycan-specific phospholipase D, with similar mobilities in the thin-layer chromatography (not shown).

The highly specific phosphatidylinositol(glycan) phospholipase C thus released water-soluble products from the lipid-isolate. These polar products were separately extracted from the silicic acid and further analyzed by column partition chromatography on a polymeric stationary phase (GlycoPak N). The three substances eluted as discrete peaks of radioactivity (Fig. 3, peak positions *I*, *II*, and *III*) (Fig. 3, *a* and *c*, the identity of peak *I*, respectively, was demonstrated by nitrous acid cleavage and partition-chromatography and by gel filtration, not shown). This identification of GlycoPak N partition-chromatography peaks with thin-layer chromatography bands was ascertained by back-chromatography on thin-layer plates of the isolated peaks (not shown). Phosphatidylinositol-glycan-specific phospholipase D-generated bands exhibited chromatographic behaviors on the GlycoPak N-column that were the same as those generated with the C-type-specific phospholipase (not shown).

A unique feature of hexosamine, with a free amino-group, in glycosidic linkage is its susceptibility to cleavage by nitrous acid deamination at pH 4. This treatment has been shown to release inositolphosphate and the shortened glycan from the well-characterized phosphoinositol glycans of protein anchors (Rosenberry et al., 1989). The three peaks from the GlycoPak N-chromatographies were separately subjected to nitrous acid treatment and analyzed by rechromatography on the same column under the same conditions (Fig. 4): the peak eluting in position *I*, corresponding to band 3 after the thin-layer chromatography, disappeared and the radioactivity instead appeared in position *IV* following nitrous acid treatment (80% yield) (Fig. 4 *a*). The other two peaks were not affected by the ni-

trous acid treatment (Fig. 4, *b* and *c*). In separate experiments we found that inositol-1-phosphate and inositol-1,2-cyclic monophosphate elute in fraction 10, hence little of this part of the phosphooligosaccharide molecule had incorporated  $^{32}\text{P}$  during a 2-h labeling of cells and that the rest of the glycan (eluting in position *IV*) contains at least one phosphate. Inositol-1,4-bisphosphate also elutes in fraction 10, demonstrating that the number of phosphate groups have little effect on the mobility of carbohydrates on the GlycoPak N column.

The three radiolabeled peaks (*I*, *II*, and *III*) from the GlycoPak N partition-chromatography were further analyzed by gel filtration on Biogel P2. The nitrous acid-sensitive substance *I* eluted with a hydrodynamic volume between that of tetraglucose and pentaglucose (Fig. 5 *a*), separate from the other two, which coeluted with higher apparent molecular mass (not shown). Nitrous acid treatment reduced the hydrodynamic volume of substance *I* to between that of tri- and tetraglucose (not shown). When the nitrous acid-sensitive (peak *I*) material was subjected to anion-exchange chromatography at pH 6, it was found to consist of three differently charged species: nonbound, eluting with 0.25 and 0.35 M buffer, respectively (Fig. 5 *b*), maybe reflecting various degrees of phosphorylation. The "middle-peak" eluting with 0.25 M buffer was variable and absent in some preparations. Mild acid treatment (1 M HCl, 30 min at 22°C or 20 mM HCl, 10 min at 100°C) of the nonbound species, caused it to bind to the anion-exchanger and then elute at 0.35 M buffer. This may indicate that the oligosaccharide with inositol cyclic phosphate, resulting from the phospholipase treatment, does not bind to the anion-exchanger. Opening of the cyclic phosphate by the acid can increase its negative charge and thus cause it to bind to the anion-exchanger. This is corroborated by an increase in the apparent hydrodynamic volume, by gel filtration, of the phosphooligosaccharide after the mild



**Figure 3.** Partition-chromatography on GlycoPak N column of water-soluble phospholipase C products. The different water-soluble products of phosphatidylinositol-glycan-specific phospholipase C hydrolysis were isolated by thin-layer chromatography (Fig. 2 *c*) and separately subjected to HPLC partition-chromatography on GlycoPak N (Methods). *I*, *II*, and *III* denote the elution positions of the respective radiolabeled products. (*a*) Band 3; (*b*) band 4; (*c*) band 2, from the thin-layer chromatography in Fig. 2 *c*.

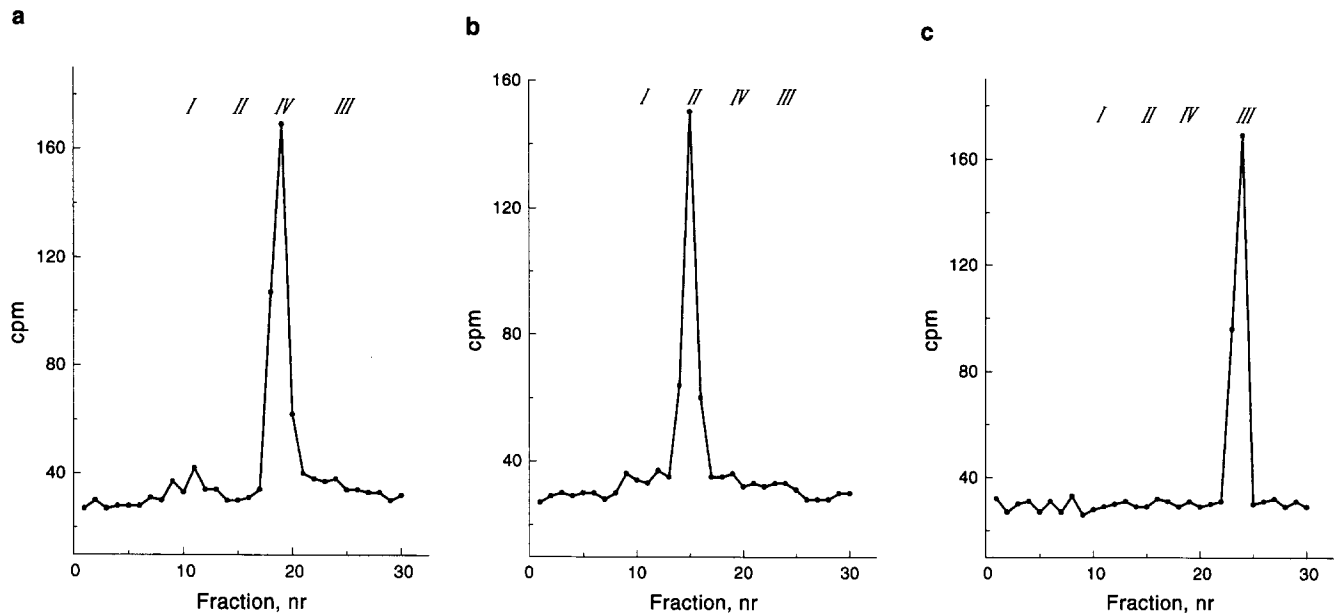


Figure 4. Partition-chromatography on GlycoPak N after nitrous acid treatment of peaks I, II, and III. The respective radiolabeled peaks after partition-chromatography of water-soluble phospholipase C products (Fig. 3) were separately treated with nitrous acid at pH 4.0 (see Materials and Methods) and rechromatographed on the GlycoPak N column. I, II, and III denote the original elution positions as in Fig. 3; IV, denotes the elution position of radiolabeled nitrous acid-treatment product. Substrates for nitrous acid treatment were (a) peak I; (b), peak II; (c), peak III, from the partition-chromatographies in Fig. 3.

acid treatment (not shown). We will refer to the nitrous acid sensitive substance I as phosphooligosaccharide.

#### Subcellular Localization of Phosphooligosaccharide

The lipid-bound form of the phosphooligosaccharide was localized to domains of the plasma membrane referred to as caveolae. These were isolated from adipocyte plasma

membranes by utilizing their insolubility in nonionic detergent and their subsequent ultracentrifugal floatation on a sucrose gradient. Isolation of such a caveolae-rich fraction was assessed by the removal of typical plasma membrane and microsomal proteins not present in caveolae and by the selective enrichment of a caveolar marker protein—caveolin. This we determined by direct comparison (by immunoblotting after SDS-PAGE) of the whole

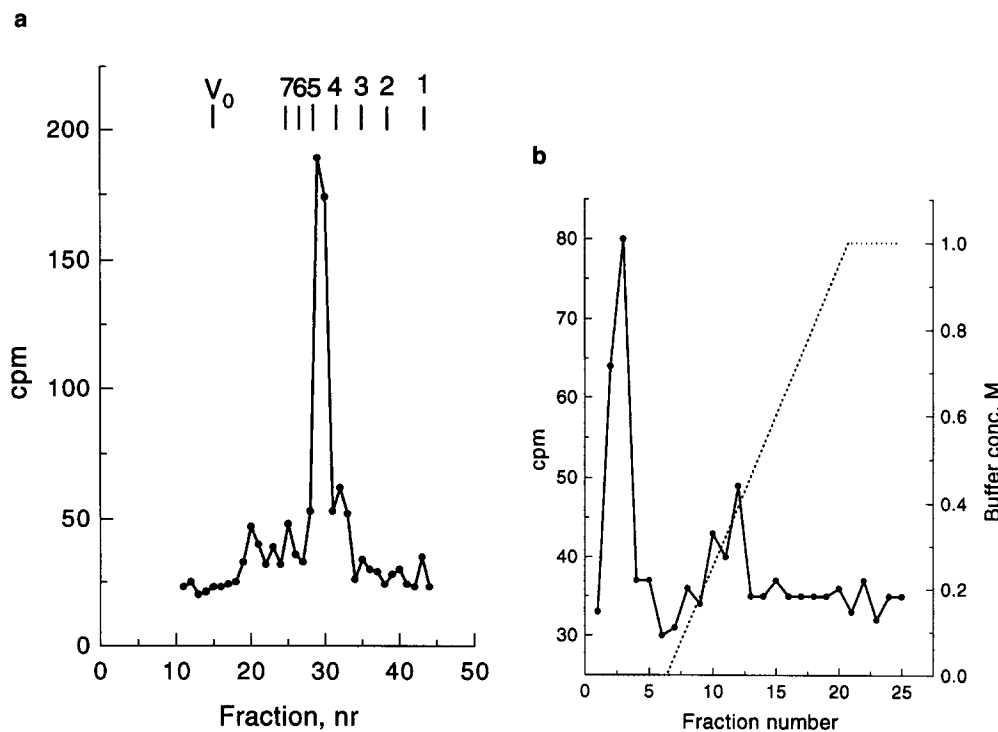


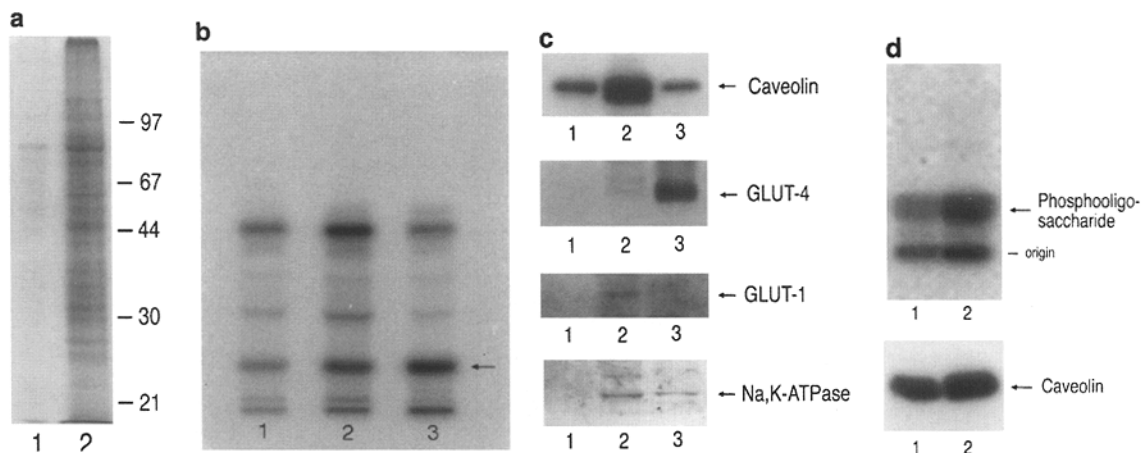
Figure 5. Gel filtration on Biogel P2 and anion-exchange chromatography of peak I from the partition-chromatography. (a) The radiolabeled material eluting in position I after partition-chromatography (Fig. 3) was subjected to gel filtration in Biogel P2 (see Materials and Methods). Indicated is the void volume ( $v_0$ ) and elution positions of oligoglucans with the indicated numbers of glucose residues. (b) The radiolabeled material eluting in position I after partition chromatography (Fig. 3) was subjected to anion-exchange chromatography on MA7P+ column (see Materials and Methods).

plasma membranes with caveolae isolated from an equal amount of plasma membranes. By extracting and purifying  $^{32}\text{P}$ -labeled phosphooligosaccharide from the same membrane preparations we analogously compared caveolae content of phosphooligosaccharide with whole plasma membrane content of phosphooligosaccharide.

We first isolated a plasma membrane enriched fraction and a microsomal fraction from [ $^{32}\text{P}$ ]phosphate-labeled adipocytes. Caveolae were then isolated by solubilization of the plasma membrane fraction with the nonionic detergent Triton X-100 and sucrose density gradient centrifugation. Triton X-100 dissolved most of the membrane proteins, <10% remained associated with the Triton X-100 insoluble caveolar fraction. That most of the plasma membrane fraction's content of proteins was solubilized and removed from the caveolar fraction is also indicated by SDS-PAGE and Coomassie blue staining. We have chosen to compare plasma membrane proteins with caveolar proteins obtained from an equal amount of plasma membranes and not by comparing equal protein loads (Fig. 6 *a*). We then analyzed for the presence of [ $^{32}\text{P}$ ]phosphooligosaccharide in the different membrane fractions and compared them by analyzing material corresponding to the same amount of starting material (Fig. 6 *b*). The identification of the thin-layer chromatography band, from the different membrane fractions, with the phosphooligosaccharide was confirmed by gel filtration behavior and susceptibility to nitrous acid deamination (not shown). Immunoblotting after SDS-PAGE compared the membrane fractions obtained from comparable amounts of starting material: anti-caveolin antibodies demonstrated the presence of caveolin in the caveolae fraction (Fig. 6 *c*), thus further identifying this detergent-insoluble preparation as

caveolae (Rothberg et al., 1992; Chang et al., 1994; Lisanti et al., 1994). To ascertain that the plasma membranes had been completely solubilized by the detergent, we assayed for the presence of glucose transporter-1 protein, a largely plasma membrane localized protein (James and Piper, 1994), and for the Na,K-ATPase alpha 2 subunit—a plasma membrane and microsomally localized protein (Shyjan and Levenson, 1989), in the different membrane fractions (Fig. 6 *c*). Glucose transporter-1 was only detected in the plasma membrane fraction and the Na,K-ATPase alpha 2 subunit in the plasma membrane and microsomal fractions, with neither protein detectable in the caveolae (Fig. 6 *c*). We also analyzed for glucose transporter-4 protein, which has been localized mainly to the microsomal fraction of non-insulin-stimulated adipocytes (James and Piper, 1994). We found this protein in the microsomal fraction and just detectable in the plasma membrane fraction (Fig. 6 *c*), thus limiting a contamination of plasma membranes by microsomes to a low level.

By densitometric scanning of chromatograms (Fig. 6, *b* and *c*) the ratio of phosphooligosaccharide to caveolin was found to be similar in the caveolae-rich fraction and in the plasma membrane preparation, indicating that much of the phosphooligosaccharide in the plasma membrane will be associated with caveolae, since caveolin is a marker for this plasma membrane structure. To further verify the presence of the phosphooligosaccharide in caveolae we prepared caveolae also from a highly purified plasma membrane preparation. The purified plasma membranes are directly compared with the caveolae-rich fraction, prepared from an equal amount of plasma membranes and not by protein load, by SDS-PAGE/immunoblotting, and by thin-layer chromatography (Fig. 6 *d*): 8% of the plasma



**Figure 6.** Subcellular localization of lipid-bound [ $^{32}\text{P}$ ]phosphooligosaccharide in adipocytes. Adipocytes were prelabeled with [ $^{32}\text{P}$ ]phosphate, homogenized and plasma membrane, caveolae, and microsomal fractions prepared as described in Methods. (*a*) SDS-PAGE (9% acrylamide) of caveolae (lane 1) and plasma membranes (lane 2), from equal amounts of cells, were stained for proteins with Coomassie blue. Indicated are the positions of molecular mass reference proteins (kD). (*b*) Subcellular fractions from equal amounts of cells were extracted and lipid-bound [ $^{32}\text{P}$ ]phosphooligosaccharide was purified by successive thin layer chromatographies, treated with phosphatidylinositol-specific phospholipase C as described and subjected to thin-layer chromatography as in Fig. 2 *c*. Arrow indicates position of phosphooligosaccharide. Lane 1, caveolae fraction; lane 2, plasma membrane fraction; lane 3, microsomal fraction. (*c*) Subcellular fractions (caveolae/plasma membrane/microsomes, 1:1:2) were subjected to SDS-PAGE and immunoblotted with monoclonal antibodies against caveolin (*Caveolin*), glucose transporter-4 (*GLUT-4*), glucose transporter-1 (*GLUT-1*), or Na,K-ATPase alpha 2 subunit (*Na,K-ATPase*). Lane 1, caveolae fraction; lane 2, plasma membrane fraction; lane 3, microsomal fraction. (*d*) Plasma membranes were further purified by density gradient centrifugation and caveolae prepared from these (see Materials and Methods). Caveolae (lane 1) and plasma membranes (lane 2), from equal amounts of cells, were analyzed as above.  $^{32}\text{P}$ -labeled phosphooligosaccharide (*top*) and caveolin (*bottom*).

membrane protein and 65% of plasma membrane caveolin was recovered in caveolae, hence there was an eightfold enrichment of caveolin in the detergent insoluble caveolae fraction compared to purified whole plasma membranes. This purification and yield of caveolin in the caveolae fraction is comparable to reported findings with chicken gizzard smooth muscle (Chang et al., 1994). For the phosphooligosaccharide there was 25% recovery in the caveolae-rich fraction and a threefold enrichment over the plasma membranes. The Na,K-ATPase alpha 2 subunit was detected by immunoblotting in the plasma membranes, but again not in the caveolae-rich fraction (not shown). The microsomal fraction (endoplasmic reticulum, golgi, endosomes, etc.) contained from one to two thirds (in different preparations) of the cellular content of lipid-bound [ $^{32}\text{P}$ ]phosphooligosaccharide (Fig. 6 b).

### Isolation of [ $^3\text{H}$ ]Phosphooligosaccharide from [ $^3\text{H}$ ]Galactose-labeled Hepatoma Cells

With hepatoma cells in culture, that were metabolically labeled with [ $^3\text{H}$ ]galactose (Fig. 1), we got the same overall results as with [ $^{32}\text{P}$ ]phosphate-labeling of adipocytes. However, in this case only a very small fraction (ca 5%) of the labeled material obtained after the successive thin-layer chromatographies was susceptible to hydrolysis by the phosphatidylinositol(glycan)-specific phospholipase C (Fig. 7), and only a fraction of thus released material represents the nitrous acid-sensitive phosphooligosaccharide (Fig. 8). When the material that partitioned into the water phase after the phospholipase treatment (Fig. 7 b, filled symbols) was subjected to the column partition-chromatography (GlycoPac N) three peaks of radioactivity were eluted (Fig. 8 a). The peaks in positions II and III corresponded to those labeled with [ $^{32}\text{P}$ ]phosphate in adipocytes (Fig. 3, b and c). The peak in fraction 9 also concealed the much smaller peak of position I (Fig. 8 a) [corresponding to the  $^{32}\text{P}$ -labeled peak in position I obtained with adipocytes (Fig. 3 a)] as is shown next.

The similarity or identity of the  $^3\text{H}$ -labeled with the  $^{32}\text{P}$ -labeled adipocyte material eluting in position I by GlycoPac N partition chromatography is demonstrated by its susceptibility to nitrous acid-cleavage, which converts both to elute at position IV (Figs. 4 a and 8 b). The majority of radiolabel peaking in fraction 9 was not affected by the nitrous acid (Fig. 8 c), demonstrating that peak I material is hidden under the comparatively large amounts of radioactivity eluting just ahead of it. The  $^3\text{H}$ -labeled materials of positions II and III were not affected by the nitrous acid (not shown), similarly to their  $^{32}\text{P}$ -labeled counterparts.

### Insulin-like Biological Activity of Phosphooligosaccharide

To test the substance eluting in position I by the GlycoPac N partition-chromatography for insulin-like effects, we prepared material from rat livers on a preparative scale and subjected it to the phospholipase hydrolysis. After column partition-chromatography we assayed the eluate for effects on protein phosphorylation in rat adipocytes. Eluting in position I we found stimulation to increased phosphorylation (by  $174 \pm 20\%$  of control, mean  $\pm$  SE,  $n = 3$ ) of a protein of apparent molecular mass 116 kD (ATP-citrate lyase) and decreased phosphorylation (to  $65 \pm 2\%$  of control, mean  $\pm$  SE,  $n = 3$ ) of 84 kD (hormone-sensitive lipase) (Fig. 9 a), characteristic of insulin action (increased to  $188 \pm 19\%$  and decreased to  $68 \pm 7\%$ , respectively, mean  $\pm$  SE,  $n = 4$ ) (Alemany et al., 1987; Strålfors and Honnor, 1989). No other fractions exhibited this effect.

Insulin also has a strong, and physiologically paramount, ability to counteract the effects of cyclic AMP elevation (Fig. 9 b) (Alemany et al., 1987; Strålfors and Honnor, 1989). This cyclic AMP counter-effect of insulin was also reproduced by the substance eluting in position I: when the adipocytes were stimulated with isoproterenol several proteins showed an increased phosphorylation (116 kD, ATP citrate lyase; 97 kD, glycogen phosphorylase; 62 kD,

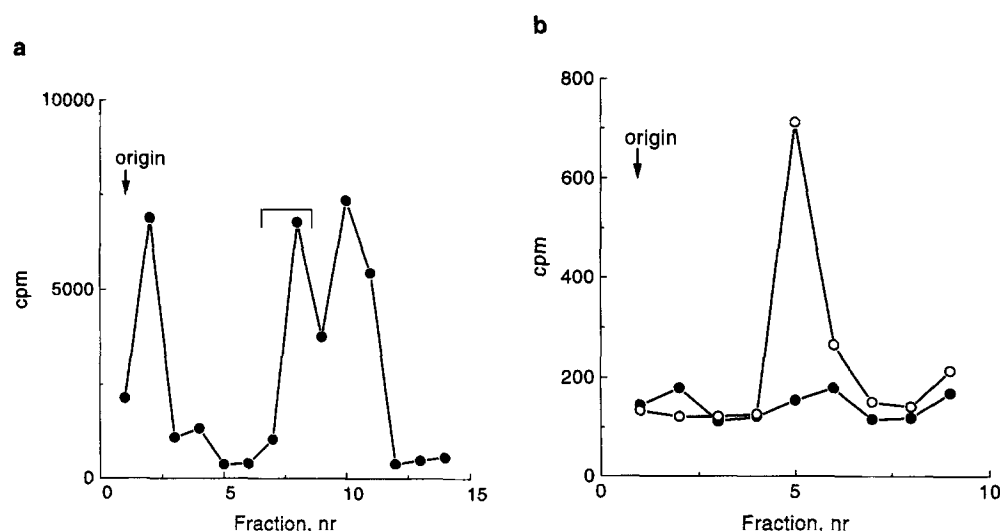
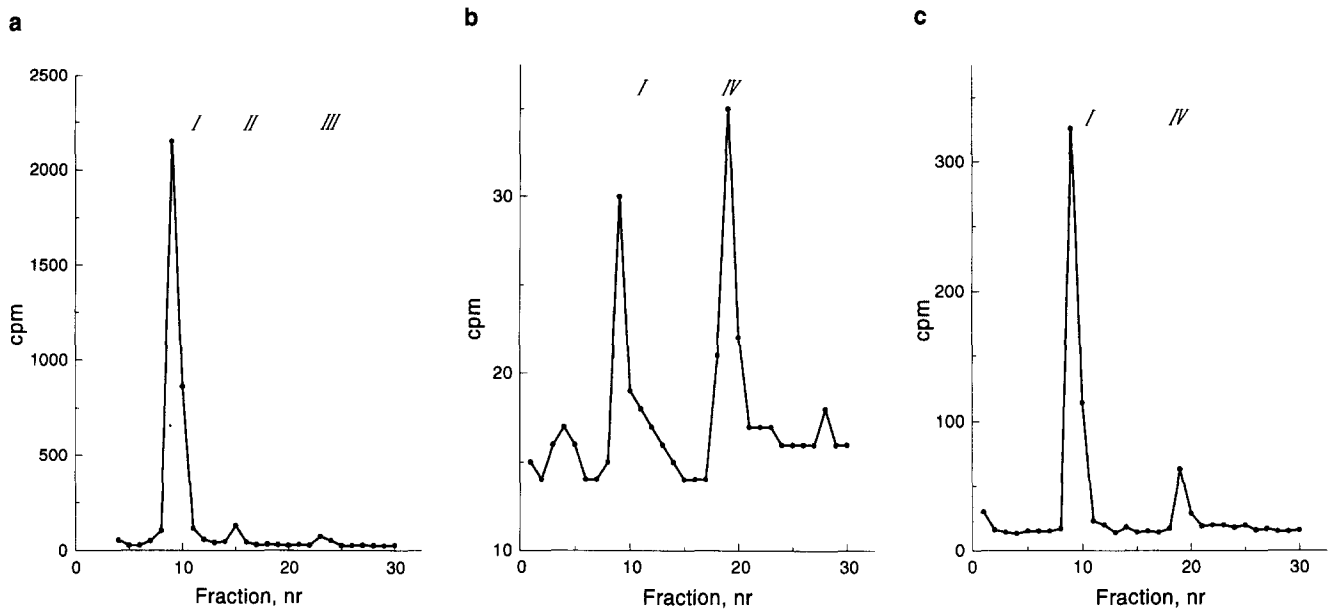
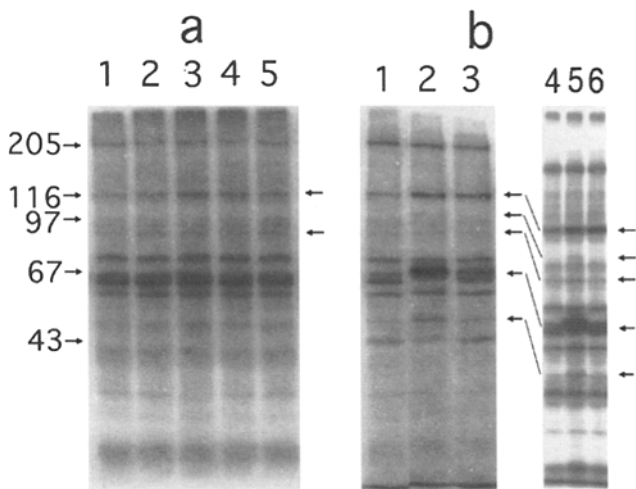


Figure 7. Thin-layer chromatography of polar lipid extract from  $^3\text{H}$ -labeled hepatoma cells. Confluent hepatoma cells were incubated with [ $^3\text{H}$ ]galactose, lipids were extracted and subjected to a first acidic thin-layer chromatography as described in Methods. (a) Second thin-layer chromatography in basic mobile phase. The indicated area was then scraped off the plate and subjected to treatment with phosphatidylinositol(glycan)-specific phospholipase C. (b) After two-phase partition water-soluble (filled symbols) and lipid-soluble (open symbols) products were separately subjected to chromatography in basic mobile phase.



**Figure 8.** Partition-chromatography on GlycoPak N column of [ $^3\text{H}$ ]galactose-labeled water-soluble products of phospholipase. (a) The water-soluble products after phosphatidylinositol(glycan)-specific phospholipase C hydrolysis were subjected to HPLC partition-chromatography on GlycoPak N (see Materials and Methods). (b)  $^3\text{H}$ -labeled peak I (fraction 11) or; (c) fraction 9-peak, were separately treated with nitrous acid at pH 4.0 (see Materials and Methods) and rechromatographed on the GlycoPak N column. I, II, III, and IV denote the elution positions of  $^{32}\text{P}$ -labeled material as in Figs. 3 and 4.



**Figure 9.** Effect of phosphooligosaccharide on the state of phosphorylation of target proteins of insulin. The phosphooligosaccharide was isolated from rat livers (see Materials and Methods) and subjected to partition-chromatography (GlycoPak N). The material eluting in position I was assayed for its insulinomimetic activity on the state of phosphorylation, in intact adipocytes, of target proteins of insulin (see Materials and Methods). Shown are autoradiographs after SDS-PAGE of total adipocyte proteins. (a) Effects of consecutive fractions on protein phosphorylation: fraction 9 (lane 1), fraction 10 (lane 2), fraction 11 = peak position I (lane 3), fraction 12 (lane 4) and fraction 13 (lane 5). (b) Effects of position I substance (fraction 11) on isoproterenol-stimulated protein phosphorylation (lane 3), control without position I substance (lane 2) and without isoproterenol or position I substance (lane 1), to be compared to control without additions (lane 4) and with isoproterenol (lane 5) and with isoproterenol plus insulin (lane 6). Indicated are the positions of the following marker pro-

teins: myosin (205 kD, unidentified protein) (Fig. 9 b). The increased phosphorylation of these was blocked by the simultaneous presence of the substance eluting in position I (Fig. 9 b). Since both cyclic AMP and insulin increase the phosphorylation of the 116 kD-protein, this effect was not blocked. Again no other fractions exhibited this property.

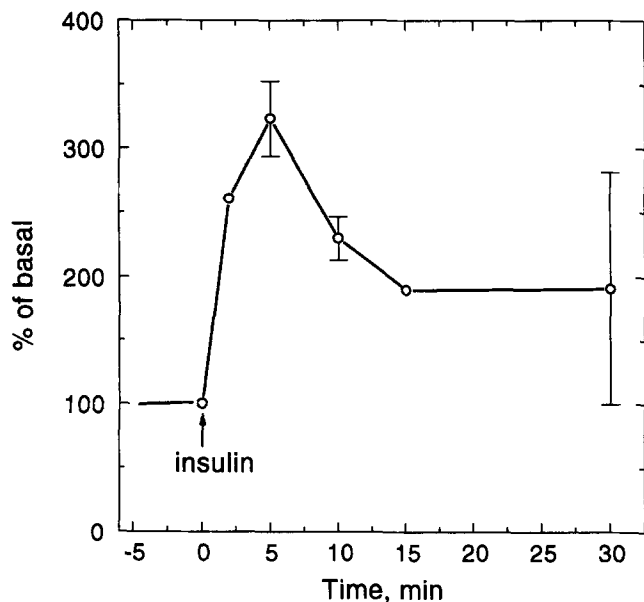
#### ***Intracellular Accumulation of Phosphooligosaccharide in Response to Insulin***

We isolated [ $^{32}\text{P}$ ]phosphooligosaccharide from the water-soluble substances of the cytosolic fraction of adipocytes, after various time periods of stimulation of the intact cells with insulin. Following gel filtration of cytosolic extracts we analyzed for the amount of [ $^{32}\text{P}$ ]phosphooligosaccharide by GlycoPak N partition-chromatography (peak position I, identified as the phosphooligosaccharide below). The intracellular level of the phosphooligosaccharide rose rapidly (within 2 min) following insulin addition to the cells and then decreased, but appeared to remain at an elevated level (Fig. 10).

We could isolate some [ $^{32}\text{P}$ ]phosphooligosaccharide also from the cell incubation medium, but if care was taken to minimize the amount of broken cells only little phosphooligosaccharide was recovered in the incubation medium. With insulin-stimulated cells: <10% of total [ $^{32}\text{P}$ ]phosphooligosaccharide was recovered in the medium of carefully

teins: myosin (205 kD),  $\beta$ -galactosidase (116 kD), glycogen phosphorylase (97 kD), bovine serum albumin (67 kD) and ovalbumin (43 kD). Also indicated are adipocyte proteins whose state of phosphorylation is affected (see text).





**Figure 10.** Time course for insulin-stimulated peak I accumulation in adipocytes. Adipocytes preincubated with [ $^{32}\text{P}$ ]phosphate were stimulated with 1 nM insulin at time zero. Samples were withdrawn before and at the indicated time-points after addition of insulin. Sampled cells were extracted as described in Methods. The cytosolic fractions were isolated, gel filtered and samples subjected to partition-chromatography on GlycoPak N, which isolated the phosphooligosaccharide from the bulk of radioactive material. Peaks of radioactivity that eluted in position I were integrated and the amount of phosphooligosaccharide expressed as % of nonstimulated value (mean of two separate experiments  $\pm$  SD).

handled cells as compared to all cells broken by ultrasonication (set to 100%). Total cytosolic volume is <1% of medium volume, hence the intracellular concentration of the phosphooligosaccharide was >1,000-fold higher than

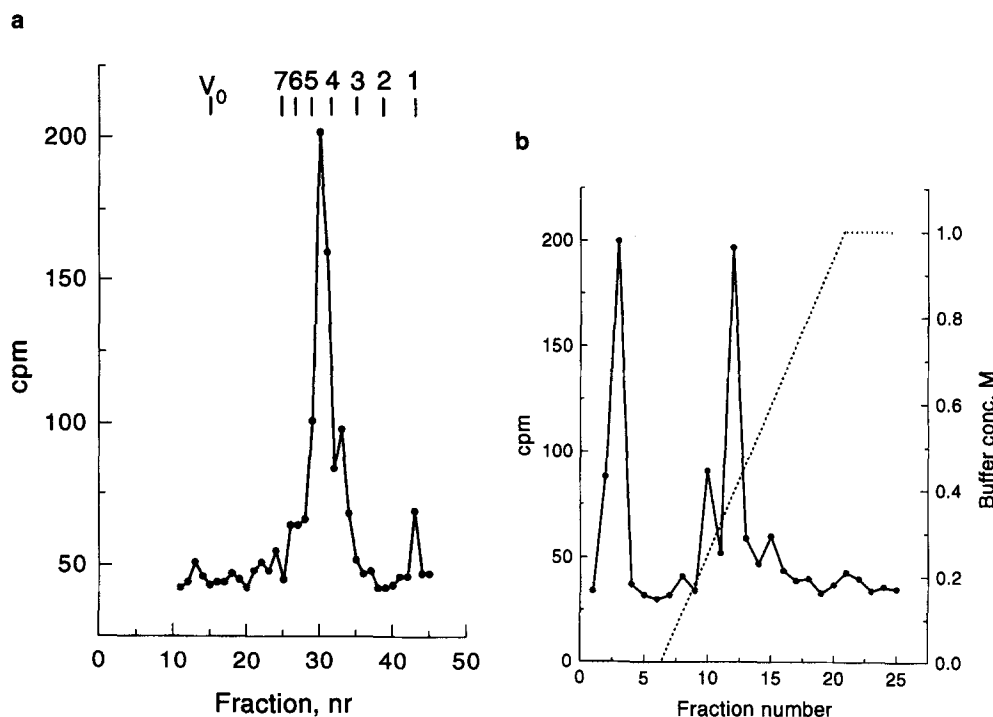
the extracellular concentration. Since it is impossible to avoid breaking some cells this indicates that much of the radiolabeled water-soluble phosphooligosaccharide recovered in the cell incubation medium was released from broken cells.

### Characteristics of Phosphooligosaccharide Generated in Response to Insulin

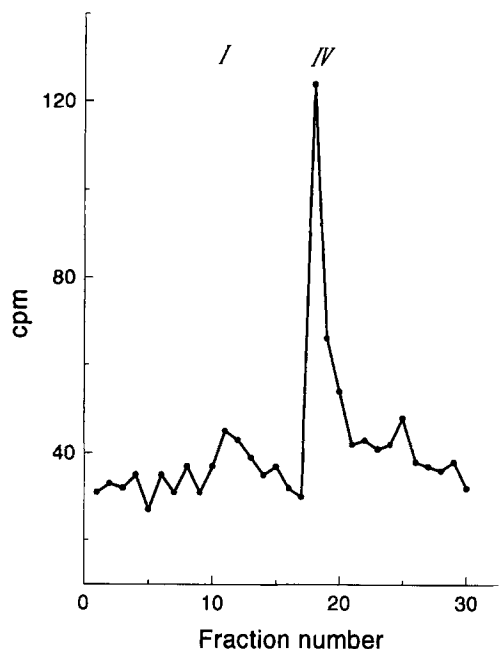
The  $^{32}\text{P}$ -labeled position I (GlycoPak N chromatography) substance from the cytosolic cell-fraction had the same chromatographic characteristics on gel filtration (Fig. 11 a) and anion-exchange chromatography (Fig. 11 b) as the phosphatidylinositol glycan-derived phosphooligosaccharide isolated from the lipid fraction of cells (Fig. 5). The "middle-peak" eluting with 0.25 M buffer by ion-exchange chromatography, was variable and absent in some preparations. Peak I obtained from cytosolic fraction of control cells or insulin-stimulated cells had the same ion-exchange chromatographic distribution (not shown), indicating that the same phosphooligosaccharides are present before and after insulin stimulation, only the amount is increased. The  $^{32}\text{P}$ -labeled phosphooligosaccharide, isolated from the cytosolic fraction of cells, was sensitive to nitrous acid deamination that caused it to elute at position IV by partition-chromatography (GlycoPak N) (Fig. 12), identically to the behavior of the lipid-derived oligosaccharide (Figs. 4 a and 8 b).

### Discussion

The findings herein conclusively demonstrate that in insulin-sensitive cells there exist phosphatidylinositol glycans, as demonstrated by phosphatidylinositol(glycan)-specific phospholipases C and D catalyzed hydrolysis and labeling with [ $^{32}\text{P}$ ]phosphate and [ $^3\text{H}$ ]galactose. A subset of these



**Figure 11.** Gel filtration and anion-exchange chromatography of peak I from the partition-chromatography of  $^{32}\text{P}$ -labeled material isolated from the cytosolic fraction of adipocytes. The radiolabeled material that eluted in position I after partition-chromatography was subjected to (a) gel filtration in Biogel P2 (see Materials and Methods). Indicated is the void volume and elution positions of oligoglucans with the indicated numbers of glucose residues. (b) The same material was subjected to anion-exchange chromatography on MA7P+ column (see Materials and Methods).



**Figure 12.** Partition-chromatography on GlycoPak N of cytosolic phosphooligosaccharide (peak *I*) after nitrous acid deamination. [ $^{32}\text{P}$ ]Phosphooligosaccharide isolated as described in Fig. 10 was treated with nitrous acid (see Materials and Methods) and rechromatographed on the GlycoPak column. *I* denotes the original elution position of untreated phosphooligosaccharide and *IV* denotes the elution position after nitrous acid deamination as defined in Fig. 4.

contains hexosamine, with a free amino-group, in glycosidic linkage and one or more phosphates. The settlement of this is critical in establishing a molecular foundation for the involvement of phosphoinositol glycan/phosphooligosaccharide in insulin action, possibly as signal transducer. These findings thus refute recent reports that such structures are nonexistent or cannot be detected after metabolic labeling of cells (Deeg et al., 1992; Whatmore et al., 1993). Our results are reproducible, although relative amounts of labeling of different peaks varied from preparation to preparation.

Our results show that the nitrous acid-sensitive phosphooligosaccharide is capable of eliciting an insulin-like response in terms of phosphorylation changes in target proteins—the ultimate event in the insulin signal transduction chain—which is a crucial property for a potential second messenger of the hormone. It is pertinent to point out here that the biological activity (in our intact cell assay system) of the phosphooligosaccharide is quite unstable (with an estimated half-life of less than one day), perhaps because the cyclic phosphate, which is the immediate product of the phospholipase C reaction, is unstable and hydrolyzes to monoesterified phosphate.

Generation and accumulation of the phosphooligosaccharide in adipocytes in response to insulin stimulation is another crucial property for a potential second messenger of the hormone. It is also a strong indication that insulin activates a phosphatidylinositol glycan-specific phospholipase in the adipocyte, since we can isolate the same substance by phospholipase treatment of a cell lipid extract (presumably from parent precursors of the insulin-released phosphooligosaccharide).

When care was exerted in handling cells, to avoid breaking them, we could only recover little of the phosphooligosaccharide from the incubation medium of the adipocytes and the intracellular concentration of phosphooligosaccharide was >1,000 times the extracellular concentration. Previous findings have suggested that the phosphooligosaccharide is generated at the cell surface (Machicao et al., 1990; Romero et al., 1990; Strålfors and Alemany, 1990; Varela et al., 1990). If this is correct, the present findings indicate that the uptake mechanism is very efficient and that the oligosaccharide is unlikely to act as a paracrine signal as has been proposed. Such an efficient uptake of insulin-generated phosphooligosaccharide can be explained by its localization to caveolae. These plasma membrane domains have been implicated in receptor and non-receptor-mediated uptake into the cytoplasm, thus avoiding the coated-pit-mediated uptake into lysosomes (Anderson et al., 1992). The localization of the lipid-bound phosphooligosaccharide to caveolae adds to the suspicion that caveolae functions as some kind of signal generating regions of the plasma membrane (Anderson et al., 1992; Sargiacomo et al., 1993; Chang et al., 1994; Lisanti et al., 1994). The localization of adipocyte insulin uptake and insulin receptors to micropinocytotic invaginations (Goldberg et al., 1987), which may represent caveolae, together with findings supporting the notion that insulin signaling occurs after receptor internalization (Kublaoui et al., 1995), offer interesting possibilities for the phosphooligosaccharide and its generation. A large fraction of  $^{32}\text{P}$ -labeled cellular lipid-bound phosphooligosaccharide was associated with the microsomal fraction, which may represent substance en route from its site of synthesis to the plasma membrane (quantitative comparisons between microsomal and plasma membrane phosphooligosaccharide cannot be made, however, since cells were labeled for 2 h only and steady-state labeling may not necessarily have been reached).

The intracellular level of the phosphooligosaccharide was rapidly elevated by insulin stimulation, reaching a peak between 2 and 5 min. It then fell over a 5-min period and appeared to remain at an elevated level. It is interesting that this is very similar to the behavior of a well-established hormonal second messenger in response to agonist stimulation: the cyclic AMP response after isoproterenol stimulation of adipocytes (Strålfors and Honnor, 1989). This time-dependent increase of intracellular phosphooligosaccharide is also compatible with the time course for insulin's effects on protein phosphorylation in adipocytes, which exhibited half-maximal effects at 5 min (Alemany et al., 1987); yet another crucial property for a potential second messenger of insulin.

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