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Ligatin Binds Phosphohexose Residues on Acidic Hydrolases

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Ligatin, a receptor that recognizes phosphorylated sugars, was isolated from plasma membranes of mouse macrophages, rat ileum, and rat brain. Several acidic hydrolases including N-acetyl β-D-glucosaminidase (β-NAG) were solubilized with this receptor. The solubilized β-NAG bound to ligatin in vitro as demonstrated by affinity chromatography using the immobilized receptor. β-N-Acetyl D-glucosaminidase–ligatin complexes were dissociated by low concentrations of mannose 6-phosphate (Man6P) and/or glucose 1-phosphate (Glc 1P). The effectiveness of these two phosphomonosaccharides varied depending on the source of the enzyme: ileal β-NAG–ligatin complexes showed a four-fold preferential dissociation with Man6P; macrophage complexes showed a 160-fold preferential dissociation with Glc 1P. Brain complexes dissociated with nearly equal preference for Man6P and Glc 1P. Heterologous complexes displayed the specificity characteristic of the source of the enzyme regardless of the source of the ligatin. Treatment of the solubilized hydrolases with endoglucosaminidase H released phosphorous-32 label from these enzymes and prevented binding of β-NAG to ligatin. However, treatment of the solubilized hydrolases with alkaline phosphatase reduced the binding of β-NAG to ligatin by no more than 30%. This apparent resistance of β-NAG to dephosphorylation was consistent with the chromatographic behavior on QAE of ^{3}H-labeled acidic oligosaccharides isolated from the solubilized hydrolases. The oligosaccharides that contain phosphorylated hexose were less acidic than phosphomonoesters and were insensitive to alkaline phosphatase until subjected to acid hydrolysis. These results suggested the presence of a phosphodiester on β-NAG analogous to the NAC glucosamine 1 P6 mannose present on β- glucuronidase isolated from mouse lymphoma cells (Tabas I, Kornfeld, S: J Biol Chem 255: 6633, 1980).

Key words: ligatin, phosphohexose, acidic hydrolases, membrane-bound receptor

Segregation of various glycoproteins to subcellular compartments has been suggested to be mediated in part by membrane-bound receptors that recognize specific carbohydrate moieties on those glycoproteins [1]. Our understanding of these vertebrate lectins has been derived primarily from functional studies [1] that show saturable binding of specific glycoproteins to cell surfaces and to isolated membranes. However, several of these vertebrate lectins have been isolated and characterized structurally as well. Two classes can be distin-

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guished. The first comprises a group of high molecular weight, integral membrane proteins that bind glycoproteins such as the mammalian hepatic asialoglycoprotein receptor [2] specific for galactose and the alveolar macrophage receptor specific for N-acetyl glucosamine and mannose [3]. The hepatic lectin mediates clearance of asialoglycoproteins from serum. The alveolar macrophage receptor binds to the cell surface mannose-terminated glycoproteins which are subsequently internalized and transported to lysosomes. The second class comprises a group of peripheral membrane proteins, namely the lactose-binding proteins [4, 5] and ligatin [6, 7]. Lactose-binding proteins are reported to be dimers with monomeric molecular weights of 12,000–13,000. These proteins appear to be distributed ubiquitously among vertebrates and may play a role in adhesion and myoblast fusion during embryonic development [4, 8]. Ligatin is a filamentous polymer comprised of monomers with an apparent molecular weight of 10,000. Ligatin binds β-N-acetyl D-glucosaminidase (β-NAG) to cell surfaces of the mammalian neonatal ileal epithelium [6], mouse peritoneal macrophages [9], and rat brain [10]. In chick embryonic neural retina, ligatin has been suggested to play a role in intercellular adhesion [7, 11]. Binding to ligatin is mediated by phosphorylated hexoses [9, 10, 25].

Acidic hydrolases are usually sequestered in lysosomes but have been identified on cell surfaces of fibroblasts [12, 13]. These enzymes include a class of glycoproteins that contain phosphorylated sugars [14–17]. Although the significance of acidic hydrolases on surfaces of fibroblasts is not well understood, it has been proposed that they are transiently exposed during the biogenesis of lysosomes. Secretion of these hydrolases is followed by endocytosis via a receptor specific for phosphorylated sugars on these cell surfaces [18]. Ligatin is a candidate for this receptor, since it recognizes phosphorylated sugars and is present at the cell surface. Additionally, ligatin may be a mechanism for targeting glycoproteins to the cell periphery, resulting in their localization at the plasma membrane. To investigate ligatin's role in the segregation of glycoproteins, we further examined the carbohydrate determinants that mediate ligatin's recognition and binding of hydrolases. We report here that β-NAG–ligatin complexes are dissociated by Man6P and/or Glc1P and, moreover, that the phosphorylated hexoses appear to be present on the protein in the form of a phosphodiester.

METHODS

Animals

C57BL/6J, C57BL/10J, and B10.D2J ♂ mice were purchased from Jackson Laboratories, Bar Harbor, ME, and used between 8 and 24 weeks of age. Neonatal (10 days of age) and adult rats (>250 gm) were purchased from Harlan/Sprague Dawley (Madison, WI).

In Vivo Labeling of Macrophages

For in vivo labeling of macrophages, 100 μCi of ^{32}P (spec. act. 10 mCi/ml, New England Nuclear, Boston, MA) was injected intraperitoneally 24 hr before harvest.

Plasma Membranes

Plasma membranes were isolated from ileal epithelial cells of neonatal rats by flotation in sucrose gradients as described previously [6]. Plasma membranes from freshly harvested thioglycollate elicited peritoneal macrophages were isolated as follows. Cells were centrifuged at 250g for 10 min and resuspended in 10 mM Tris, 1 mM $MgCl_2$, 1 mM KCl, pH 7.4, buffer [19]. The cell suspension was frozen and thawed twice and then diluted with an equal volume of 10 mM Tris, pH 8.1, buffer. Freeze thawing of the diluted suspension was repeated once.

The plasma membranes were isolated by centrifugation at 95,000g for 45 min on discontinuous sucrose gradients consisting of 26, 36, and 40% (w/w) sucrose in 10 mM Hepes, 1 mM NaN_3, pH 7.6, buffer (Hepes buffer). Membranes at the 0/26 and 26/36% sucrose interfaces were pooled, diluted with Hepes buffer and collected by centrifugation at 30,000g for 20 min. Identification of this material as predominantly plasma membranes was established by enzymatic assay for 5′ nucleotidase [20] and aminopeptidase [21] activities and by electron microscopy. Mitochondrial membranes and some lysosomal membranes were present in the 36/40% sucrose interface. Other lysosomal membranes and cellular debris were pelleted during the 95,000g centrifugation.

Plasma membranes were isolated from rat cerebra using a modification of the synaptosomal preparation of Cotman and Matthews [22]. Cerebra were dissected so as to remove the olfactory lobes and the brain stem posterior to the superior colliculus. Tissue was homogenized (two cerebra/25 ml) in 10 mM Hepes, 1 mM $MgCl_2$, 1 mM NaN_3, 0.32 M sucrose, pH 7.3, buffer (0.32 M sucrose–Hepes buffer) in a Dounce homogenizer (8–10 strokes by hand) and centrifuged at 330g for 5 min. The pellet (P1) was retained and the supernatant recentrifuged at 14,500g for 15 min. The pelleted material (P2) was collected and the supernatant discarded. P1 and P2 were resuspended separately in 5 vol of 0.32 M sucrose–Hepes buffer and fractionated on step gradients containing 7.5 and 13% ficoll in 0.32 M sucrose–Hepes buffer by centrifugation at 110,000g for 60 min. Material at the 7.5/13% ficoll interface was collected, resuspended by homogenization in 20 vol of 10 mM Hepes, 1 mM $MgCl_2$, 1 mM NaN_3 pH 7.3 buffer (Hepes–Mg buffer) and pelleted by centrifugation at 32,500g for 15 min. Resuspension in Hepes–Mg buffer and centrifugation of the pelleted membranes were repeated once. Enrichment of this material for plasma membranes was determined by enzymatic assays for 5′ nucleotidase and alkaline phosphatase activities and by electron microscopy. Although enriched for plasma membranes, mitochondrial and lysosomal membranes were also present as determined by electron microscopy and acid phosphatase activity.

Isolation of Ligatin and Associated Hydrolases

Plasma membrane pellets were treated with 40 mM $CaCl_2$ to release ligatin–ligand complexes and then dialyzed against 5 mM Hepes, 0.5 mM EGTA, pH 8.0, buffer overnight (4°C) to depolymerize ligatin and dissociate the ligatin–ligand complex. The dialysate was centrifuged at 110,000g for 90 min to remove the extracted membranes. The supernatant was chromato-

graphed on a Biogel P100 column (BioRad Laboratories, Rockville, NY, 1 × 20 cm, flow rate 3 ml per hr) equilibrated in Hepes buffer to separate monomeric ligatin from the ligand. The ligatin preparation was further purified by dialysing it against 3 mM $CaCl_2$ to form polymers and then rechromatographing it on a Biogel P100 column. Exclusion of the ligatin polymers from the Biogel P100 matrix was confirmed by negative stain electron microscopy. Purity of the various ligatins was confirmed by gel electrophoresis in which ligatin migrated as a single polypeptide. No other polypeptides were resolved by conventional staining procedures [6, 9].

The ligatin-consolubilized hydrolases isolated from brain and macrophages were applied to DEAE–cellulose columns. The peak containing β-NAG and aminopeptidase activities from macrophages [9] and the peak containing β-NAG and acetylcholinesterase activities from brain [10, 25] were used.

Biochemical Determinations

Protein was determined by the procedure of Lowry et al [23] using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as the standard. β-N-Acetyl D-glucosaminidase activity (5–25 units/ml) that had been solubilized with ligatin was assayed using p-nitrophenyl β-N-acetylglucosaminide (3 mM, Sigma) in 0.2 ml of 0.2 M sodium citrate, pH 4.5, for 5 min to 8 hr at 37°C [24]. Enzymatic reactions were terminated by the addition of two parts of 1 M K_2CO_3. Where indicated, activities were converted to units of micromoles p-nitrophenol released per minute, using a molar extinction coefficient of 1.24×10^4. Acid phosphatase [26] and alkaline phosphatase activities were measured analogously using 4 mM p-nitrophenyl phosphate (Sigma) in either 0.15 M Na acetate pH 5.5 for acid phosphatase or 10 mM Hepes, pH 7.6, for alkaline phosphatase.

Affinity Chromatography

Two hundred to 500 μg of ligatin were covalently coupled to 1 to 2 ml of Affi-gel 10 resin (Bio-Rad Laboratories). Derivitization was effected at 23°C for 2 hr in 0.1 M Na phosphate, pH 7.0, buffer. Unreacted active esters were saturated by incubation at 23°C with 0.1 M glycine, pH 7.0 buffer, for 1 hr.

The affinity column was equilibrated in 10 mM Hepes, 3 mM $CaCl_2$, 50 mM NaCl, 1 mM NaN_3, pH 7.6, buffer. Sodium or potassium salts of phosphohexoses, nucleosides, nucleoside phosphates, and nonphosphorylated sugars were purchased from Sigma Chemical Co. Five column volumes of these sugars were used for elution (see Results for concentrations). Alkaline phosphatase (calf intestine) was purchased from Boehringer Mannheim (Indianapolis, IN). Coupling of alkaline phosphatase (30 units) to Affi-gel 10 resin was performed as described previously. The ligatin-cosolubilized hydrolases were incubated with alkaline phosphatase–Affi-gel 10 in 10 mM Hepes buffer for 24 hr (37°C). The ligatin-cosolubilized hydrolases were then separated from the alkaline phosphatase by sedimentation.

Removal of N glycosidically linked oligosaccharides from the ligatin-cosolubilized hydrolases was done using 10 munits of endoglucosaminidase H (Streptomyces griseus, Miles Chemical Co. Elkhart, IN) in 25 mM NH_4 Ac, pH 5.5, buffer for 12 hr (37°C). Freed oligosaccharides were separated from the ligatin-cosolubilized hydrolases by chromatography on a Biogel P100 column.

QAE–Sephadex Fractionation

The N glycosidically linked oligosaccharides released by endoglucosaminidase H from ligatin-cosolubilized hydrolases were ^{3}H-labeled and reduced with tritiated borohydride [16]. The ^{3}H-oligosaccharides were then fractionated on QAE–Sephadex (Sigma Q-25-120) equilibrated in 2 mM Tris base as described by Tabas and Kornfeld [17]. Bound material was eluted stepwise with various concentrations of NaCl in 2 mM Tris base (see Results for concentrations). Half-milliliter fractions were collected and counted for radioactivity. The elution positions of ^{3}H-mannose 6-phosphate and ^{3}H-mannitol 6-phosphate were at 100 mM NaCl.

Enzyme Digestions

Aliquots of the ^{3}H-labeled oligosaccharides were digested in 0.05 ml of Vibrio choleral neuraminidase (Sigma, 70 units/ml) in 25 mM ammonium acetate, pH 5.5, for 24 hr at 37°C. Tritiated oligosaccharides were also digested in 0.03 ml of pig liver α-N acetylglucosaminidase (gift of Dr. S. Kornfeld, 2.7 units/ml) in 50 mM sodium citrate, pH 4.5, for 24 hr at 37°C and in 0.05 ml Escherichia coli alkaline phosphatase (Sigma, 40 units/ml) in 50 mM Tris, pH 8.0, buffer for 24 hr at 37°C.

Acid Hydrolysis

Oligosaccharides were heated at 100°C for 30 min in 0.1 ml of 0.1 N HCl (pH 2.5) [17]. The samples were then frozen, lyophilyzed, and resuspended in 2 mM Tris base for QAE–Sephadex fractionation.

Paper Electrophoresis

The ligatin-cosolubilized glycoproteins from ileum, brain, and macrophages were hydrolyzed with 20 mM HCl for 18 hr in the presence of Dowex 50W. The hydrolysate was subsequently reduced and tritiated with tritiated borohydride according to the procedure of Distler et al [11]. The ^{3}H-labeled monosaccharides were applied to a fresh Dowex-50W column (0.5 × 3 cm) from which they were eluted with 15 ml of water. This effluent was then applied to a DEAE–cellulose column (in bicarbonate) to enrich for the acidic monosaccharides. The DEAE–cellulose-bound material was subsequently eluted with 0.5 M ammonium bicarbonate, pH 8.5, neutralized, dried under reduced pressure, and subjected to paper electrophoresis in pyridine acetate (1.25 M pyridine, 64 mM acetic acid, pH 6.4). The chromatogram was run at 14 V/cm for 90 min. A single peak of radioactivity was observed to comigrate with phosphohexose standards in pyridine acetate. The migration of this radioactive peak was altered following its digestion with alkaline phosphatase (6 units, 18 h, 37°C) to that of a neutral glycitol.

RESULTS

Affinity Chromatography

The procedure used for isolating ligatin from ileal epithelial plasma membranes also results in the selective release of β-NAG [6]. In macrophages [9] and in brains [10], this procedure solubilizes a set of hydrolases including

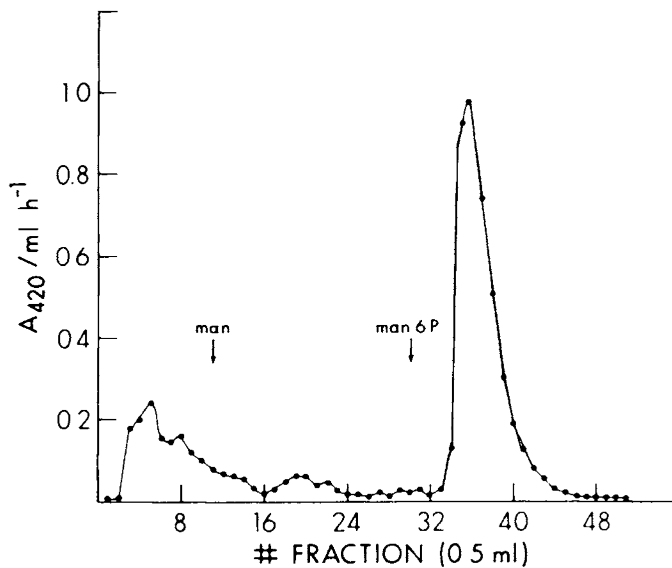

Fig. 1. Elution profile from a ligatin–Affi-gel 10 column. Forty-five units of β-N-acetyl D-glucosaminidase (0.5 ml) isolated from ileal epithelial plasma membranes were applied. Unbound ileal β-N-acetyl D-glucosaminidase was eluted with 10 mM Hepes, 3 mM $CaCl_2$, 50 mM NaCl, 1 mM NaN_3, pH 7.6, buffer. Four percent of bound β-N-acetyl D-glucosaminidase activity eluted with mannose (40 mM in the above buffer); 96% with mannose 6-phosphate (40 mM in the above buffer). Left ordinate is the specific activity of β-N-acetyl D-glucosaminidase expressed as A_{420} units per milliliter of sample per hour.

β-NAG. To investigate whether or not the solubilization of these hydrolases with ligatin was coincidental, affinity chromatography using a ligatin-derivatized matrix was used. Two hundred to 500 μg of ligatin were immobilized on 1 to 2 ml of Affi-gel 10 resin. Lower amounts of immobilized ligatin resulted in minimal or no binding, suggesting a multivalency in binding [27]. Application of the ligatin-cosolubilized proteins to the ligatin-affinity column resulted in the adsorption of β-NAG. A typical elution profile is shown in Figure 1. β-N-Acetyl D-glucosaminidase could be released from the affinity column by phosphohexoses (0.5 to 40 mM). Nonphosphorylated sugars (40 and 100 mM) and inorganic phosphate (40 mM) had minimal or no effect, suggesting the presence of a phosphorylated hexose on the enzyme (see below). Among the phosphorylated sugars used, a range of efficacy in releasing bound β-NAG from the ligatin affinity column was observed. Furthermore, the effectiveness of the phosphomonosaccharides varied depending on the source of the enzyme: ileal β-NAG complexes showed a four-fold preferential dissociation with Man6P (Table I), macrophage complexes showed a 160-fold preferential dissociation with Glc 1P (Table II). Brain complexes dissociated with nearly equal preference for Man6P and Glc 1P (Table III). Heterologous complexes displayed the specificity characteristic of the source of the enzyme.

TABLE I. Release of Ileal β-N-Acetyl D-Glucosaminidase From Ligatin-Affi-Gel 10 Matrix*

Treatment[a]	Concentration (mM)	Released (%)
A. Sugar and sugar phosphates[b]		
Mannose 6-phosphate	0.5	46.0
Mannose 1-phosphate	0.5	0.0
Glucose 6-phosphate	0.5	0.0
Glucose 1-phosphate	0.5	0.0
Glucosamine 6-phosphate	0.5	14.0
Mannose 6-phosphate	5.0	55.0
Mannose 1-phosphate	5.0	0.0
Mannose 6-phosphate	20.0	60.0
Glucosamine 6-phosphate	20.0	50.0
Mannose 6-phosphate	40.0	90.0
Mannose 6-phosphate	40.0	100.0
Mannose 1-phosphate	40.0	0.0
Glucose 6-phosphate	40.0	22.0
Glucose 1-phosphate	40.0	50.0
Glucosamine 6-phosphate	40.0	59.0
Galactose 1-phosphate	40.0	59.0
Fructose 1, 6-diphosphate	40.0	21.0
Fructose 1-phosphate	40.0	79.0
Mannose	40.0	4.4
Glucose	40.0	0.0
Glucosamine	40.0	12.0
Galactose	40.0	0.0
Fructose	40.0	0.0
B. Nucleosides and nucleoside phosphates		
Adenosine monophosphate	40.0	7.1
Adenosine diphosphate	40.0	2.0
Uridine diphosphate	40.0	18.7

*Forty-five units of β-N-acetyl D-glucosaminidase isolated from ileal epithelial plasma membranes were applied in 0.5 ml. Specific activity of this enzyme was 65–77 μmol split/mg min^{-1}.
[a]Na or K salts in 10 mM Hepes, 1 mM NaN_3, 3 mM $CaCl_2$, 50 mM NaCl, pH 7.6, buffer.
[b]NAC-glucosamine 1-phosphate (3 mM), uridine diphosphoglucose (5 mM), and uridine diphospho-NAC-glucosamine (3 mM) at 6- to 10-fold higher concentrations than mannose 6-phosphate (0.5 mM) were only 4–35% as effective in elution.

As a control for the ligatin affinity column, β-NAG was applied to glycine-saturated Affi-gel 10 resin. Binding was minimal compared with binding to a ligatin-derivatized column. Maximally only 10% of the applied β-NAG bound to a glycine-saturated resin, while greater than 80% bound to the ligatin-derivatized matrix, demonstrating the specificity of the column.

To determine if a phosphorylated hexose on β-NAG was recognized by ligatin and accessable to alkaline phosphatase, the enzyme was incubated with phosphatase (24 hr, 37°C) before affinity chromatography. The alkaline phosphatase was first covalently linked to Affi-gel 10 matrix to enable its removal from the ligatin-cosolubilized proteins. Unexpectedly, the alkaline phosphatase digestion of these proteins did not alter the binding of the β-NAG to the ligatin matrix by more than 30%, and did not alter its elution by phospho-sugars. This

TABLE II. Release of Macrophage β-N-Acetyl D Glucosaminidase From Ligatin-Affi-Gel 10 Matrix*

Treatment[a]	Concentration (mM)	Released (%)
A. Sugar and sugar phosphates[b]		
Glucose 1-phosphate	0.5	99.0
Glucosamine NAC 1-phosphate	0.5	8.5
Mannose 6-phosphate	0.5	0.0
Glucose 1-phosphate	40.0	91.0
Glucose 6-phosphate	40.0	11.0
Mannose 1-phosphate	40.0	14.0
Mannose 6-phosphate	40.0	43.0
Fructose 1-phosphate	40.0	17.0
Inorganic phosphate	40.0	0.0
Glucosamine	40.0	5.4
Galactose	40.0	10.3
Glucose	100.0	0.5
Mannose	100.0	4.2
Fructose	100.0	8.0
B. Nucleoside and nucleoside phosphates		
Adenosine monophosphate	10.0	12.0
Adenosine diphosphate	10.0	12.8
Adenosine triphosphate	10.0	18.0
Uridine diphosphate	10.0	17.9
Cytosine diphosphate	10.0	13.0

*A half unit of β-N-acetyl D-glucosaminidase isolated from plasma membranes of macrophages were applied in 0.5 ml.

[a]Na or K salts in 10 mM Hepes, 1 mM NaN_3, 3 mM $CaCl_2$, 50 mM NaCl, pH 7.6, buffer.

[b]Galactose 1-phosphate (10 mM), galactose 6-phosphate (10 mM), and glucosamine 6-phosphate (20 mM) at 20- and 40-fold higher concentrations than glucose 1-phosphate (0.5 mM) were only 10–30% as effective in elution.

minimal effect on the binding of β-NAG to ligatin was not due to an inactivation of the alkaline phosphatase by coupling it to Affi-gel, as measured in control experiments by p-nitrophenol release from p-nitrophenyl phosphate. However, it may have resulted from an inaccessibility of this moiety to the immobilized phosphatase.

As an alternative approach to determine if a phosphorylated sugar was present on the β-NAG, we treated the enzyme with endoglucosaminidase H prior to affinity chromatography. Phosphorylated hexose(s) on acidic lysosomal hydrolases have been reported to be located on N-glycosidically linked oligosaccharides of the high mannose type that can be removed by endoglucosaminidase H [14, 15]. Macrophages were labeled in vivo for 24 hr with inorganic phosphorus-32 in order to monitor dephosphorylation. The ^{32}P-labeled macrophages were subsequently harvested and the ligatin-cosolubilized β-NAG was isolated. Phosphorus-32-labeled material containing β-NAG activity was treated with endoglucosaminidase H (12 hr, 37°C) and chromatographed on a Biogel P100 column to separate freed oligosaccharides from the β-NAG. The excluded fraction from the Biogel P100 column that contained β-NAG activity had retained only 16% of the ^{32}P label. When this was chromatographed on a

TABLE III. Release of Brain Hydrolases From Ligatin–Affi-Gel 10 Matrix*

Treatment[a]	Concentration (mM)	Released[b]
Glucose 1-phosphate	3.0	20.0
Mannose 6-phosphate	3.0	30.0
Glucose 1-phosphate	10.0	30.0
Glucose 6-phosphate	10.0	4.0
Mannose 1-phosphate	10.0	4.0
Mannose 6-phosphate	10.0	30.0
Galactose 6-phosphate	10.0	<6.0
Glucose 1-phosphate	20.0	63.0
Mannose 6-phosphate	20.0	55.0
Glucosamine	20.0	20.0
Glucose 1-phosphate	40.0	77.0
Mannose 6-phosphate	40.0	55.0
Glucosamine 1-phosphate	40.0	<10.0
Galactose 6-phosphate	40.0	<6.0
Fructose 1-phosphate	40.0	50.0
Fructose 6-phosphate	40.0	14.0
Galactose	40.0	<6.0
Glucose	40.0	<20.0
Mannose	40.0	<20.0
Fructose	40.0	12.0

*Two to five units of β-N-acetyl D-glucosaminidase isolated from plasma membranes of brain were applied in 0.2 ml. In some instances, iodinated ligatin cosolubilized material was applied to the affinity column and was monitored by scintillation counting. No differences in the elution profiles of the iodinated material and of β-N-acetyl D-glucosaminidase activity was observed.
[a]Na or K salts in 10 mM Hepes, 1 mM NaN_3, 3 mM $CaCl_2$, 50 mM NaCl, pH 7.6, buffer.
[b]^{125}I label or β-N-acetyl D-glucosaminidase activity.

ligatin affinity column, binding reduced to 22% relative to the untreated enzyme, indicating that the phosphorylated sugar recognized by ligatin was located on N glycosidically linked oligosaccharide(s). The amount of ^{32}P eluted from this column was too low to determine whether the binding of treated β-NAG was mediated by residual phospho-sugar. Similar results were obtained with endogeneously labeled brain hydrolases [25]. These studies were not done with the ileal β-NAG.

QAE-Sephadex Fractionation

Our results from affinity chromatography suggested that phosphomonosaccharide residues, specifically Glc 1P and Man6P, mediated binding of β-NAG to ligatin. Furthermore, digestion with endoglucosaminidase H of those ^{32}P-labeled hydrolases that cosolubilized with ligatin resulted in the release of the ^{32}P label and reduction of the binding of β-NAG to ligatin. This suggested that β-NAG contained phosphorylated hexose residues on N glycosidically linked oligosaccharide(s). Yet, digestion of the β-NAG with alkaline phosphatase only minimally altered its binding to the ligatin affinity column. Since Tabas and Kornfeld have demonstrated that newly synthesized oligosaccharides

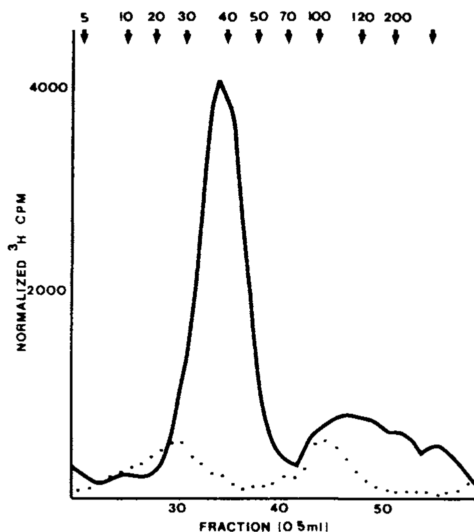

Fig. 2. QAE–Sephadex fractionation of endoglucosaminidase H released oligosaccharides from ileal, macrophage, and brain ligatin-cosolubilized hydrolases. Released oligosaccharides were ^3H labeled and reduced with tritiated borohydride. After acid hydrolysis the elution position of the major oligosaccharides changed to show a decrease in net negative charge as evidenced by a decrease in NaCl concentration required for elution. Digestion with alkaline phosphatase after acid hydrolysis greatly reduced binding to QAE-Sephadex. Untreated (——). Alkaline phosphatase-treated after acid hydrolysis (----). Concentration (mM) of NaCl applied is indicated by arrows.

of β-glucuronidase contain phosphatase resistant phosphate in a phosphodiester linkage, we reasoned that the resistance to alkaline phosphatase of the β-NAG isolated from ileum, macrophage, and brain may be due to similar linkages. To demonstrate the presence of a negatively charged oligosaccharide(s) on these hydrolases and to determine whether a phosphodiester linkage was present, the endo-glucosaminidase H-sensitive oligosaccharides from the hydrolases were isolated for analysis (see Methods). The oligosaccharides were subsequently ^3H labeled by reduction with tritiated borohydride. The ^3H-labeled oligosaccharides were then fractionated on QAE-Sephadex. A typical elution profile is shown in Figure 2. The dominant peak of bound radioactivity eluted at 30 mM NaCl and in some instances migrated as a doublet at 30–40 mM NaCl. Mannitol 6-phosphate and mannose 6-phosphate eluted at 100 mM NaCl.

Alkaline phosphatase digestion of the ^3H-oligosaccharides isolated from macrophage and brain hydrolases did not change the net negative charge of the oligosaccharides that eluted at 30 mM NaCl, in that no loss of binding to QAE–Sephadex was observed (Table IV). Only 30% of the ^3H-labeled oligosaccharides from ileal β-NAG were dephosphorylated by this treatment. This resistance to alkaline phosphatase was consistent with our previous observations using affinity chromatography and with the presence of a phosphodiester. To investigate the latter, we subjected aliquots of the negatively charged ^3H-labeled oligosaccharides to a limited acid hydrolysis (pH 2.5, 100°C, 30 min) to

TABLE IV. Effect of Various Treatments on Acidic Oligosaccharides*

A. Source	Applied not bound to QAE–Sephadex (%)			
	No treatment	After ALKP[a]	After 0.1 N HCl	After 0.1 N HCl followed by ALKP[a]
Macrophage	32	31	67	87
Brain	47	52	62	92
Ileum	95	97	96	99

B. Source	Bound that eluted in 30 mM NaCl (%)[b]					
	No treatment	After neuraminidase	After ALKP[a]	After α HEX[a] followed by ALKP[a]	After 0.1 N HCl	After 0.1 N HCl followed by ALKP[a]
Macrophage	100	90	96	92	41	19
	(1.86)	(1.69)	(1.79)	(1.68)	(0.77)	(0.35)
Brain	100	100	91	80	65	15
	(0.41)	(0.41)	(0.38)	(0.33)	(0.27)	(0.06)
Ileum	100	89	58	39[c]	71	21
	(3.39)	(3.02)	(1.95)	(1.30)	(2.41)	(0.70)

*QAE–Sephadex fractionation of endoglucosaminidase H released oligosaccharides from ileal, macrophage, and brain ligatin-cosolubilized hydrolases. The released oligosaccharides were ^3H labeled and reduced by reaction with tritiated borohydride. The indicated treatments and the QAE–Sephadex fractionation were carried out as described under Methods. The percentage (^3H) cpm not bound to the QAE–Sephadex was calculated from the fraction of total cpm applied. Total cpm of ^3H-labeled oligosaccharides applied to each of these QAE–Sephadex columns were as follows: 7.2 (10^5) cpm from ileal β-N-acetyl D-glucosaminidase, 9.6 (10^3) cpm from brain ligatin-cosolubilized hydrolases, and 3.2 (10^4) cpm from macrophage ligatin-cosolubilized hydrolases.
[a]Abbreviations used: alkaline phosphatase (ALKP); α-N-acetylglucosaminidase (a-HEX).
[b]Given in parenthesis is cpm bound × 10^4.
[c]Eluted in 20 mM NaCl.

cleave the postulated one linkage of the diester and expose a phosphomonoester. The behavior of the ^3H-labeled oligosaccharides on QAE/Sephadex and the susceptibility of these oligosaccharides to alkaline phosphatase were then reexamined. No increase in the net negative charge of the ^3H-labeled oligosaccharides was observed, in that the concentration of NaCl required to elute them from QAE–Sephadex did not increase. Instead, a decrease in their net negative charge was observed, most notably in the oligosaccharides isolated from ileal β-NAG. Furthermore, susceptibility to alkaline phosphatase could now be demonstrated (see Figure 2, Table IV). Digestion of the ^3H-labeled oligosaccharides with neuraminidase did not alter their behavior on QAE–Sephadex, indicating that the observed decrease in their net negative charge after a limited acid hydrolysis was not due to removal of sialic acid.

In an attempt to identify the acid labile moieties blocking phosphatase digestion of the ^3H-labeled phospho-oligosaccharides, the ^3H-labeled oligosaccharides were incubated for 24 hr with pig liver α-N-acetylglucosaminidase. The removal of the blocking moiety was then assayed by testing the susceptibility of the α-N-acetylglucosaminidase-treated oligosaccharides to alkaline phospha-

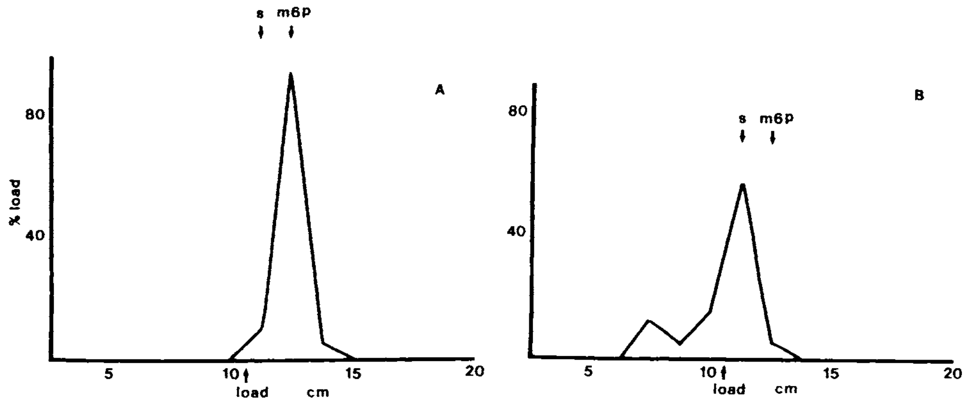

Fig. 3. Paper electrophoresis of tritiated borohydride reduced acidic monosaccharides isolated from macrophage ligatin-cosolubilized hydrolases in pyridine acetate. Migration of untreated (A) and alkaline phosphatase treated material (B) is shown relative to sorbitol(s) and mannitol 6-phosphate (M6P).

tase. Twenty percent of the ileal acidic oligosaccharides which were previously insensitive to phosphatase were now dephosphorylated (Table IV) to give a 60% reduction in binding as compared to the untreated ^{3}H-labeled oligosaccharides. Likewise, 11% of the acidic oligosaccharides from brain and 4% of the acidic oligosaccharides from macrophage became susceptible to dephosphorylation after digestion with α-N-acetylglucosaminidase. This partial susceptibility to α-N-acetylglucosaminidase suggests that the blocking moieties of at least some of the phosphorylated oligosaccharides present on ligatin-cosolubilized hydrolases from ileum, brain, and macrophages are α-N-acetylglucosamine residues.

Paper Electrophoresis

The QAE–Sephadex data demonstrated that the major negatively charged oligosaccharides isolated from brain, macrophages, and ileal epithelium contain covalently bound phosphate residues. However, these phosphate residues are not sensitive to alkaline phosphatase prior to limited acid hydrolysis suggesting the presence of an acid-labile "blocking" moiety. Further evidence of the presence of phosphate within these oligosaccharides was obtained by paper electrophoresis. The ligatin-cosolubilized glycoproteins from ileum, brain, and macrophage were hydrolyzed with 20 mM HCl for 18 hr in the presence of Dowex 50W. The hydrolysate was subsequently reduced and tritiated with tritiated borohydride (see Methods). The ^{3}H-labeled monosaccharides were then applied to a Dowex 50W column, eluted with water, and then applied to a DEAE–cellulose column to enrich for the acidic monosaccharides. The DEAE–cellulose-bound material was subsequently eluted, neutralized, dried under reduced pressure, and subjected to paper electrophoresis in pyridine acetate. A single peak of radioactivity was observed to comigrate with phospho-hexose (Fig. 3a). The migration of this radioactive peak was altered following its digestion with alkaline phosphatase to that of a neutral glycitol (Fig. 3b).

DISCUSSION

A receptor for hydrolases that contain phosphorylated sugars has been purified from plasma membranes of neonatal rat ileal epithelial cells [6], thioglycollate-activated mouse peritoneal macrophages [9], and rat cerebrum [10, 25]. Although not identical, these proteins are closely related in that they possess the following properties common to all ligatins: they are solubilized from membranes by Ca^{2+} and are depolymerized by EGTA; they form filaments 3 nm in diameter in 3 mM Ca^{2+}; they have an absorption maximum at 255 nm due to disulfides (manuscript in preparation); they do not precipitate in polyacrylamide gels with conventional fixatives; they have aparent monomeric molecular weights of ~10,000; they have atypically low contents of aromatic and hydrophobic residues; and they have specificity for phosphorylated hexoses.

The procedure used for isolating ligatin from plasma membranes also results in the solubilization of several hydrolases. Of these hydrolases, a high specific activity of β-NAG is present. The isolated β-NAG from ileal epithelial cells has a specific activity of 65–77 μmol split/mg min^{-1} and accounts for greater than 90% of the protein detected by polyacrylamide gel electrophoresis [6]. This is comparable to specific activities reported for β-NAG purified from pig kidney (54.5 and 39.9 μmol split/mg min^{-1}) [28] and from human liver (81.0 and 51.7 μmol split/mg min^{-1}) [28]. The β-NAG isolated with ligatin from macrophages and brain have lower specific activities, 16.2 μmol split/mg min^{-1} and 25 μmol split/mg min^{-1}, respectively. From these two sources other hydrolases (including aminopeptidase from macrophages and acetylcholinesterase from brain) are solubilized. The ligatin-cosolubilized aminopeptidase and acetylcholinesterase bind to the ligatin-derivatized matrices and can be eluted from the ligatin affinity columns by Glc 1P and Man 6P.

The solubilization of β-NAG with ligatin is not coincidental. Instead, affinity chromatography using a ligatin-derivatized matrix demonstrates that ligatin is capable of binding this enzyme. The ligatin–hydrolase complex can be dissociated by low concentrations of certain phosphorylated hexoses, in particular Man 6P and Glc 1P. This suggests a highly specific phosphohexose-mediated binding. A simple cation bridging of phosphate residues between the phosphorylated sugars and immobilized ligatin is not solely responsible for in vitro binding since adsorption of β-NAG and its release by phosphohexoses (0.5–40 mM) occurs in the presence of 40 mM phosphate and in the absence of cations (unpublished results). Furthermore, stereospecificity among the phosphorylated sugars used to compete in vitro for binding is observed. Mannose 6P is most effective in releasing bound ileal β-NAG from a ligatin Affi-gel matrix. This contrasts with the elution profiles observed with the macrophage and brain hydrolases wherein Glc 1P is either several fold more effective or equally effective as Man6P in competing for bound enzyme. Nonphosphorylated sugars, nucleosides, nucleoside phosphates, and inorganic phosphate have minimal or no effect. Incubation of ^{32}P-labeled ligatin-cosolubilized material that contains β-NAG activity with endoglucosaminidase H removes the ^{32}P label and prevents the binding of β-NAG to ligatin, again suggesting that the recognition site on this hydrolase includes phosphorylated hexose residues on N glycosidically linked oligosaccharides.

The existence of negatively charged oligosaccharide(s) sensitive to endoglucosaminidase H digestion was demonstrated by chromatography on QAE–Sephadex. These studies also confirmed the resistance of those negatively charged oligosaccharide(s) to alkaline phosphatase. Following a limited acid hydrolysis, the oligosaccharides exhibited an altered behavior on QAE–Sephadex and a susceptibility to alkaline phosphatase. The latter behavior is consistent with the removal of a 1-glycosidic linkage resulting in exposure of a phosphomonoester. However, the behavior of these negatively charged oligosaccharides on QAE–Sephadex following removal of the "blocking moiety" was to elute as a more neutral species, behavior unlike that of the phosphodiester present on β-glucuronidase isolated from mouse lymphoma cells [12]. This decrease in net negative charge of the acidic oligosaccharides after a limited acid hydrolysis was not due to removal of sialic acid since digestion with neuraminidase did not change their behavior on QAE–Sephadex. Our results are consistent with a "blocked" phosphorylated sugar present on these acidic oligosaccharides, although their migration may be influenced by additional factors such as the position of modifying groups on the oligosaccharides.

Removal of the blocking moiety by digestion of the ^{3}H-labeled oligosaccharides from ileal and brain hydrolases with pig liver αN-acetylglucosaminidase suggests that at least some of the phosphorylated sugar present on these enzymes is blocked with α-N-acetylglucosamine. The α-N-acetylglucosaminidase-resistant oligosaccharides of macrophages, brain, and ileum could have either other types of blocking moieties such as glucose or α-N-acetylglucosaminidase-resistant α-N-acetylglucosamine. The identity of the "blocking moieties" on the α-N-acetylglucosaminidase-resistant oligosaccharides of macrophages, brain, and ileum is currently being pursued.

The location of ligatin and its associated hydrolases on cell surfaces may result from a transient fusion of lysosomes with the plasma membrane. Our data suggest otherwise in that the amount of ligatin present on the cisternal surfaces of macrophage lysosomes is small relative to that found on the cell periphery [9], although selective extraction may account for this apparent distribution. Distinction between a lysosomal versus a cell surface location for ligatin is not trivial in that both intracellular [29] and extracellular [18, 30] membrane bound receptors specific for phosphorylated sugars have been postulated to sequester lysosomal hydrolases during biogenesis of lysosomes. In the latter reports, the derivatized sugar is a phospho-monoester, Man 6P [15, 16].

Although the significance of acidic hydrolases on cell surfaces is not understood, the presence of a receptor specific for phosphorylated sugars provides a mechanism for targeting particular glycoproteins to membranes. Once bound, the glycoprotein can subsequently be sequestered to an intracellular compartment as occurs in receptor-mediated endocytosis. Alternatively, these glycoproteins can remain sequestered at the cell periphery. Receptors, such as ligatin, that recognize a defined post-translational modification provide a generalized mechanism for compartmentization. Differential synthesis of these glycoproteins during differentiation could thus result in changes in function of the cell surface without necessitating changes in the receptor.

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