Characterization of the mannose 6-phosphate-dependent pathway of lysosomal enzyme routing in an invertebrate

Vivian ALVAREZ, Armando J. PARODI* and Roberto COUSO†

Instituto de Investigaciones Bioquímicas 'Fundación Campomar', Antonio Machado 151, 1405 Buenos Aires, Argentina

The mannose 6-phosphate (Man6P)-dependent pathway for routing lysosomal enzymes was characterized in the hepatopancreas of the estuary crab *Chasmagnatus granulata*: (a) an acid α -L-fucosidase was purified to homogeneity from the above-mentioned organ and was shown to contain mannose-linked phosphate residues; (b) high-mannose-type oligosaccharides isolated from a protein fraction enriched in acid hydrolases were found to contain acid-labile *N*-acetylglucosamine (GlcNAc) residues; (c) a membrane-bound UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase was detected that phosphorylated the estuary-crab α -L-fucosidase and bovine uteroferrin but not bovine pancreas ribonuclease B; (d) a GlcNAc-1-

phosphodiester α -*N*-acetylglucosaminidase that released GlcNAc units from GlcNAc α 1-P6Man α 1-methyl was detected in microsomal membranes of the hepatopancreas; (e) two detergent-solubilized microsomal proteins having molecular masses of 205 and 215 kDa that were retained by a Man6P-rich mannan-Sepharose column, from where they were eluted with Man6P but not with glucose 6-phosphate, were recognized by antisera raised against bovine large (215 kDa) and small (46 kDa) Man6P receptors. This is the first description of all the components of the Man6P-dependent mechanism for routing lysosomal enzymes in an invertebrate.

INTRODUCTION

Two different mechanisms for routing lysosomal enzymes may be operative in mammalian cells, one of them dependent on the presence of mannose 6-phosphate (Man6P) markers in the enzymes and the other independent of the presence of such residues [1–3]. The latter is exclusively based, as are those that are operative in fungal and plant cells, on particular features of the protein domains of lysosomal enzymes [4,5]. In the Man6P-dependent mechanism, the first step in the formation of those markers involves the transfer of *N*-acetylglucosamine (GlcNAc)-1-*P* units from UDP-GlcNAc to position 6 of α 1–2-linked mannose units in high-mannose-type oligosaccharides [6]. The transferring enzyme (UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase) specifically recognizes certain features in the protein domain of lysosomal enzymes, since it efficiently phosphorylates such enzymes but not non-lysosomal glycoproteins having similar high-mannose-type oligosaccharides [7]. The phosphotransferase has been localized in the *cis* cisternae of the Golgi apparatus [8]. The following step, the removal of the GlcNAc units, is catalysed by a GlcNAc-1-phosphodiester α -*N*-acetylglucosaminidase that does not show any specific structural requirement in the protein moiety for efficient sugar removal [9]. Two receptors for Man6P markers, having molecular masses of 215 and 46 kDa, have been found in mammalian cells [10,11]. Clathrin-coated vesicles containing those receptors are responsible for the transport of lysosomal enzymes from the *trans*-Golgi cisternae or the plasma membranes to endosomes [12].

UDP-GlcNAc:glycoprotein GlcNAc-1-phosphotransferases have been described in lower eukaryotes. The enzyme from the cellular slime mould *Dictyostelium discoideum* differs from that described in mammalian cells because it phosphorylates the

lysosomal enzyme cathepsin D and non-lysosomal glycoproteins, such as ribonuclease B or ovalbumin, with the same low efficiency [13]. Surprisingly, another lysosomal enzyme, uteroferrin, is recognized by the *D. discoideum* enzyme as a very good substrate. It was postulated that this was due to a special conformation of the oligosaccharide in uteroferrin and not to the recognition of a structural feature in the protein backbone by the phosphotransferase, because the deglycosylated uteroferrin failed to inhibit phosphorylation of the glycosylated species. In *D. discoideum* lysosomal enzymes only 2–3 % of the Man6P residues are in the form of monoesters. The majority are methylphosphate mannose diesters [14]. In fact, targeting of lysosomal enzymes in *D. discoideum* is not mediated by Man6P markers [15]. A UDP-GlcNAc:glycoprotein GlcNAc-1-phosphotransferase with specificity, with respect to lysosomal enzymes, similar to that of the mammalian enzyme has been described in the unicellular organism *Acanthamoeba castellanii* [16]. However, other components of the routing mechanism (a GlcNAc-1-phosphodiester α -*N*-acetylglucosaminidase and Man6P receptors) could not be detected in this micro-organism.

The Man6P-dependent mechanism for lysosomal enzyme routing has been described to occur not only in mammalian but also in avian and reptile cells [17]. Up to now a description of all its main components in invertebrates has been lacking. A UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase with the same specificity, with respect to lysosomal enzymes, as that from mammalian cells was detected in the fruitfly *Ceratitis capitata* [18]. The presence of other components was not investigated. On the other hand, a recent report showed that a UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase, phosphorylated high-mannose-type oligosaccharides and Man6P receptors were absent from insect Sf9 cells [19]. In the present

paper we describe for the first time the presence of all the components of the routing mechanism in an invertebrate.

EXPERIMENTAL

Materials

Concanavalin A-Sepharose, Sepharose 4B and the Mono Q HR 5/5 and Superose 12 HR 10/30 columns were from Pharmacia (Uppsala, Sweden). UDP-[^3H]GlcNAc (5–25 Ci/mmol), UDP-[^{14}C]GlcNAc (220 Ci/mol), Na ^{125}I , NaB $^3\text{H}_4$ (12 Ci/mmol) and [γ - ^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear (Boston, MA, U.S.A.). QAE-Sephadex, *Streptomyces griseus* protease type XIV (Pronase), *Escherichia coli* alkaline phosphatase, all protein inhibitors mentioned in the text, *Staphylococcus aureus* Protein A, Trizma, bovine pancreatic ribonuclease B, protein molecular-mass standards, all *p*-nitrophenyl derivatives, α -methyl mannoside, mannitol, Man6P and glucose 6-phosphate (Glc6P) were from Sigma (St. Louis, MO, U.S.A.). Iodo-Gen (1,3,4,6-tetrachloro-3 α -6 α -diphenylglycoluril) was from Pierce (Rockford, IL, U.S.A.).

Preparations

[β - ^{32}P]UDP-GlcNAc was prepared using [γ - ^{32}P]ATP as described by Lang and Kornfeld [20]. GlcNAc α 1-[^{32}P]P6Man α 1-methyl or [^{14}C]GlcNAc α 1-P6Man α 1-methyl were prepared using [β - ^{32}P]UDP-GlcNAc or UDP-[^{14}C]GlcNAc, respectively, and α -methyl mannoside as substrates and the UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase from *A. castellani* as previously described [13]. A mild acid treatment (0.01 M HCl for 15 min at 100 °C) of GlcNAc α 1-[^{32}P]P6Man α 1-methyl produced [^{32}P]P6Man α 1-methyl. *Hansenula holstii* (strain NRRL-Y-2448) phosphomannan was prepared, treated with mild acid and coupled to Sepharose 4B as already described [21–23]. Rabbit antisera raised against 215 and 46 kDa bovine Man6P receptors were generously given by S. Kornfeld (Division of Hematology/Oncology, Washington University School of Medicine, St. Louis, MO, U.S.A.). Reduction of GlcNAc and Man6P with NaBH $_4$ produced *N*-acetylglucosaminitol and mannitol 6-*P* respectively.

Chasmagnatus granulata

This estuary crab was collected at Samborombón Bay (Province of Buenos Aires, Argentina). The crabs were killed and the hepatopancreas immediately extracted. They were frozen in liquid nitrogen and kept at –70 °C until used.

Purification of α -L-fucosidase

(a) Homogenization

The hepatopancreases (about 30 in a typical preparation) were ground in a mortar cooled with liquid nitrogen. The resulting powder was resuspended in 50 mM Tris/HCl buffer, pH 7.0, containing 10 mM sodium β -glycerophosphate, 1 mM PMSF, 1 mM 1-phenyl-2-thiourea and 1 mM butylhydroxytoluene and sonicated 5 $\times$ 30 s at 200 W in a Braun Sonic 1510 machine. The resulting protein concentration was 25–30 mg/ml.

(b) (NH $_4$) $_2$ SO $_4$ precipitation

The suspension was centrifuged at 12000 *g* for 15 min. The supernatant was then centrifuged at 100000 *g* for 45 min, and that supernatant was adjusted to 80 % (w/v) (NH $_4$) $_2$ SO $_4$ con-

centration. The precipitate was removed after centrifugation at 12000 *g* for 15 min. The supernatant was discarded.

(c) Acid extraction

The (NH $_4$) $_2$ SO $_4$ precipitate was resuspended in 100 mM sodium acetate buffer (pH 4.0)/10 mM sodium β -glycerophosphate/1 mM PMSF and centrifuged at 12000 *g* for 15 min. The supernatant was removed and the precipitate re-extracted as above. The supernatants were pooled.

(d) Affinity chromatography

A volume of 1 M Trizma equal to one-fifth the volume of supernatant was added to it. The pH of the solution was adjusted to 8.0 with 10 M NaOH. CaCl $_2$, MgCl $_2$ and MnCl $_2$ were added to 1 mM and NaCl to 0.15 M final concentrations respectively. The sample was twice applied (elution rate 0.3 ml/min) to a 10 ml concanavalin A-Sepharose column equilibrated with 10 mM Tris/HCl buffer (pH 8.0)/0.15 M NaCl/10 mM sodium β -glycerophosphate/CaCl $_2$ + MnCl $_2$ + MgCl $_2$ (1 mM each) (buffer A). The column was then washed with 50 volumes of buffer A and the acid hydrolase was eluted with 0.5 M α -methyl mannoside in buffer A. Fractions of 1 ml were collected. Those with higher activity were pooled and dialysed against 50 mM Tris/HCl buffer, pH 7.6, and 10 mM sodium β -glycerophosphate (buffer B).

(e) Ion-exchange chromatography

The preparation was then submitted to ion-exchange chromatography in a Mono Q HR 5/5 column in a FPLC machine. The column was equilibrated with buffer B. The sample was applied at a rate of 1 ml/min. Elution was performed with 30 ml of a 0–0.5 M NaCl gradient in buffer B and 15 ml of a 0.5–1 M NaCl gradient in the same buffer. Fractions of 1 ml were collected and those with higher activity were pooled and dialysed against 50 mM Tris/HCl buffer (pH 8.0)/10 mM sodium β -glycerophosphate/0.15 M NaCl (buffer C).

(f) Gel-filtration chromatography

The preparation was concentrated to 0.5 ml by ultrafiltration and submitted to gel-filtration chromatography in a FPLC machine by using a Superose 12 HR 10/30 column equilibrated with buffer C. The elution rate was 0.5 ml/min. Fractions of 0.5 ml were collected, and those with higher activity were pooled and concentrated to 0.5 ml as above. The preparation was submitted a second time to gel-filtration chromatography as above.

Only one protein band of molecular mass 57 kDa appeared when the final preparation was submitted to 10 % (w/v) PAGE under denaturing conditions. The gel was stained with silver nitrate and the same single band appeared, even when the gel was overloaded. The α -L-fucosidase was very stable, and the yield of a typical preparation was 30–35 %. The enzyme appeared to be homogeneous after a 7500-fold purification.

Enzymic assays

All enzymes were assayed at 22 °C except where indicated. Acid hydrolases were assayed using the respective *p*-nitrophenyl derivatives as described previously [24]. The UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase was assayed essentially as described by Reitman et al. [25] by using [β - ^{32}P]UDP-GlcNAc and α -methyl mannoside as substrates and 120 μg of membrane

proteins. When α -L-fucosidase, uteroferrin or ribonuclease B were used as acceptors, the concentration of the sugar nucleotide was 140 μ M and the radioactivity per assay was 5×10^6 c.p.m. Reactions were stopped by the addition of 1 ml of 5 mM NaEDTA, pH 7.8. Proteins were precipitated with 20% (w/v) trichloroacetic acid and the precipitates were twice washed with cold acetone and counted. The GlcNAc-1-phosphodiester α -N-acetylglucosaminidase was assayed in incubation mixtures containing, in a total volume of 30 μ l, 4×10^5 c.p.m. of [14 C]GlcNAc α 1-P6Man α 1-methyl, 50 mM Tris/maleate buffer, pH 6.8, 10 mM GlcNAc, 5 mM EDTA and 90 μ g of membrane proteins. Incubations were performed at 22 °C and were stopped by the addition of 2 ml of 2 mM Trizma. The suspensions were applied to 1 ml QAE-Sephadex columns equilibrated with 2 mM Trizma. Non-retained substances were counted for radioactivity or concentrated, desalted and submitted to paper chromatography as indicated in the text.

Preparation of hepatopancreas extracts for enzymic and receptor assays

The hepatopancreases were homogenized as described in the first step of the α -L-fucosidase purification procedure. The homogenate was centrifuged at 12000 *g* for 15 min. The supernatant was then centrifuged at 100000 *g* for 45 min. For measuring the UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase the resulting pellet was homogenized in 50 mM Tris/HCl buffer, pH 7.5, containing 2% (v/v) Lubrol PX, 0.5% (w/v) sodium deoxycholate, 2 mM PMSF and 20 mg/ml of each of leupeptin, antipain, pepstatin, E64 and chymostatin. For assaying the GlcNAc-1-phosphodiester α -N-acetylglucosaminidase the membrane pellet was resuspended in 5 mM sodium maleate buffer (pH 6.8)/0.1% (v/v) Triton X-100/10 mM Man6P. The suspension was sonicated for 30 s at 50 W. The final protein concentration was 25 mg/ml. The suspension was kept at 4 °C for 2 h and centrifuged at 160000 *g* for 45 min. The pellet was suspended as above but without Man6P. The pellet obtained upon a second high-speed centrifugation was homogenized in 5 mM sodium maleate buffer, pH 6.8, containing 1% (v/v) Triton X-100, 2 mM PMSF and 20 μ g/ml of each of leupeptin, antipain, pepstatin, E64 and chymostatin. The final protein concentration was 30 mg/ml. The suspension was centrifuged at 160000 *g* for 60 min and the supernatant was used as the enzyme source. For isolating the Man6P receptors the membrane pellet was resuspended in 50 mM Tris/HCl buffer, pH 7.0, containing 0.15 M NaCl, 0.5% (w/v) saponin, 5 mM sodium β -glycerophosphate, 10 mM Man6P, 0.34 units/ml Trasylol, and 20 μ g/ml of each of leupeptin, antipain, pepstatin, E64 and chymostatin. After 1 h at 4 °C the membrane suspension was centrifuged at 100000 *g* for 45 min. The pellet was four times resuspended and centrifuged as above, twice in the presence and twice in the absence of Man6P. The pellet was finally resuspended in 50 mM Tris/HCl buffer, pH 7.2, containing 0.15 M NaCl, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, 0.34 units/ml Trasylol, and 20 μ g/ml of each of leupeptin, antipain, pepstatin, E64 and chymostatin (buffer D). After 1 h at 4 °C, the suspension was centrifuged at 100000 *g* for 45 min. The supernatant was used as the receptor source.

Purification of Man6P receptors

The extract (1 ml) prepared as described above was 10-fold diluted with buffer D without detergent and the solution adjusted to 10 mM of each of $MnCl_2$, $MgCl_2$ and $CaCl_2$. It was then applied to a 1 ml column of a phosphomannan-Sepharose 4B column equilibrated with 50 mM Tris/HCl buffer, pH 7.2, con-

taining 0.15 M NaCl, 0.05% (v/v) Triton X-100, 5 mM sodium β -glycerophosphate, 10 mM of each of $MnCl_2$, $MgCl_2$ and $CaCl_2$, 0.34 units/ml Trasylol, and 20 μ g/ml of each of leupeptin, antipain, pepstatin, E64 and chymostatin (buffer E). After 2 h, 200 ml of buffer E, 20 ml of buffer E containing 2 mM Glc6P and 20 ml of buffer E containing 2 mM Man6P were successively applied to the column. Proteins present in the eluting solutions were precipitated with 20% (w/v) trichloroacetic acid. The pellets were washed with cold acetone and subjected to 7% or 10% (w/v) PAGE in the presence of SDS.

Methods

(a) Reduction with NaB^3H_4

Samples were dried and dissolved in 0.1 ml of 0.2 M NaOH and 0.1 M sodium borate buffer, pH 10.3. NaB^3H_4 (1 mCi) was added. The incubation lasted for 2 h at 37 °C after which 1 ml of 1 M HCl was added. The solution was dried and 1 ml of methanol was added and dried. This procedure was repeated four times more. The sample was then dissolved in water and applied to a 5 ml Dowex 50 (H^+ form) column. Non-retained material was dried, and the addition of methanol and evaporation were performed five more times.

(b) Proteolytic degradation of proteins

Proteins were heated for 20 min at 100 °C. The precipitate obtained after centrifugation at 10000 *g* for 15 min was resuspended in 1 ml of 0.1 M Tris/HCl buffer, pH 8.0 and 1 mM $CaCl_2$ containing 40 mg of *S. griseus* protease. The protease had been previously heated in the same solution for 30 min at 60 °C. After 16 h at 55 °C, the solution was heated for 10 min at 100 °C and the supernatant obtained after centrifugation at 10000 *g* for 15 min was desalted by gel-filtration chromatography through a 60 cm $\times$ 1 cm Sephadex G-10 column equilibrated with 7% (v/v) propan-2-ol.

(c) Affinity chromatography of glycopeptides

This was performed essentially as described above for the α -L-fucosidase purification but with a 5 ml lectin column. The samples were desalted and the α -methyl mannoside removed by gel-filtration chromatography as described above.

(d) Paper chromatography and electrophoresis

They were performed on Whatman 1 papers with the following solvents: A, butan-1-ol/ethanol/water (4:1:1, by vol.); B, 1.2 M pyridine acetate, pH 6.5; C, propan-2-ol/acetic acid/water (27:4:9, by vol.); D, nitromethane/ethanol/acetic acid/boric acid-saturated water (8:1:1:1, by vol.); E, 1% (w/v) sodium tetraborate; F, butan-1-ol/pyridine/water (10:3:3, by vol.); and G, ethanol/acetic acid/1 M ammonium acetate (75:26:4, by vol.). Electrophoresis was performed at 20 V/cm.

(e) Labelling of *S. aureus* Protein A

This was performed by using $Na^{125}I$ and Iodo-Gen and standard procedures.

RESULTS AND DISCUSSION

Lysosomal enzymes contain mannose-linked phosphate units

Several acid hydrolases (β -galactosidase, β -glucosidase, α -glucosidase, β -D-N-acetylglucosaminidase, α -mannosidase and α -L-

Figure 1 Mannose-linked phosphate units are present in *C. granulata* α -L-fucosidase

The α -L-fucosidase purified from about 30 hepatopancreases was degraded with a non-specific protease; the high-mannose-type glycopeptides were isolated and submitted to strong acid hydrolysis (4 h at 100 °C in 1 M HCl). The resulting material was reduced with NaB^3H_4 , partially purified by paper chromatography in solvent A and by paper electrophoresis in solvent B, and assayed by paper chromatography in solvent C (a). The substance migrating as the mannitol 6-*P* standard was eluted and assayed by paper electrophoresis in solvent B (b). The substance in the peak close to the Man6*P* standard was eluted, treated with alkaline phosphatase and assayed again by paper electrophoresis in solvent B (c). The substance running as a mannitol standard in (c) was eluted and assayed by paper chromatography in solvent D (d) or by paper electrophoresis in solvent E (e). Chromatography and electrophoresis products were scanned in a paper scanner. For further details see the text. Standards: 1, mannitol 6-*P*; 2, glucuronitol; 3, glucose; 4, UMP; 5, Man6*P*; 6, mannitol; 7, galactitol; and 8, sorbitol.

fucosidase) were detected when a crude extract of the hepatopancreas of the estuary crab *C. granulata* was incubated with the corresponding *p*-nitrophenyl derivatives. The highest activity was shown by the α -L-fucosidase, which was then purified to homogeneity as described in the Experimental section. The enzyme appeared to have a molecular mass of 57 kDa under denaturing conditions, to have asparagine-linked oligosaccharides, as it was retained by a concanavalin A-Sepharose column from which it was eluted with 0.5 M α -methyl mannoside, and to have a broad optimum pH, ranging between 3 and 5.

The α -L-fucosidase was degraded with an unspecific protease and the resulting high-mannose-type glycopeptides were isolated by affinity chromatography on a concanavalin A-Sepharose

column. The glycopeptides were then submitted to strong acid hydrolysis (4 h at 100 °C in 1 M HCl). Under these conditions more than 85 % of Man6*P* remains unaffected whereas glycosidic bonds are completely degraded [26]. The degradation products were reduced with sodium [^3H]borohydride and assayed by paper chromatography in solvent A. In this solvent neutral monosaccharides migrate whereas charged ones remain at the origin. Substances at the origin were eluted and submitted to paper electrophoresis in solvent B. Substances migrating as or near a Man6*P* standard were then submitted to paper chromatography in solvent C (Figure 1a). The substance migrating as a mannitol 6-*P* standard was eluted and submitted again to paper electrophoresis in solvent B. The majority of the labelled substance migrated as a negatively charged substance, close to the position of a Man6*P* standard (Figure 1b). The charge was removed when the labelled substance was eluted from the paper [Figure 1(b)] and treated with alkaline phosphatase (Figure 1c). The neutral substance thus produced ran as a mannitol standard during paper chromatography in solvent D (Figure 1d) and during paper electrophoresis in solvent E (Figure 1e). The analytical systems employed clearly showed that the phosphate units were linked to mannose and not to glucose or galactose residues. Although the position to which the phosphate units were linked was not established, it may be assumed that, similarly to what occurs in mammalian cells, the compound formed in α -L-fucosidase was Man6*P*.

Presence of acid-labile GlcNAc residues in a protein extract enriched in acid hydrolases

As mentioned above, in mammalian cells the first step in the formation of Man6*P* markers is the transfer of GlcNAc-1-*P* to mannose units. The GlcNAc residues are later removed. It was assumed that if transfer of GlcNAc-1-*P* occurred in *C. granulata*, some GlcNAc residues could still be linked by acid-labile bonds in acid hydrolases, provided that an incomplete enzymic removal occurred *in vivo*. The protein extract eluted with α -methyl mannoside from concanavalin A-Sepharose (see the purification procedure of α -L-fucosidase in the Experimental section) was found to be enriched in all acid hydrolases mentioned above. The extract was degraded with an unspecific protease and high-mannose-type glycopeptides were isolated by affinity chromatography through a concanavalin A-Sepharose column. Half the glycopeptides were treated with mild acid (0.01 M HCl for 10 min at 100 °C). Both halves were then reduced with NaB^3H_4 . The reaction products were assayed by paper chromatography in solvent A. A very high background without any noticeable peak in the position where a *N*-acetylglucosaminitol standard migrated appeared in both samples. Nevertheless, labelled substances migrating as the above-mentioned standard were eluted and assayed by paper chromatography in solvent E. A high background was still observed in both samples, but the acid-treated one showed a slight peak in the position of *N*-acetylglucosaminitol. Substances migrating as a *N*-acetylglucosaminitol standard were eluted and re-run in the same solvent. As shown in Figure 2, only the acid-treated sample showed a peak in the position of *N*-acetylglucosaminitol. This result strongly suggests, but does not prove, that acid hydrolases might contain GlcNAc-1-*P* residues.

The hepatopancreas of the estuary crab *C. granulata* has an UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase

The corresponding mammalian enzyme recognizes α -methyl mannoside as an acceptor, although with a very low affinity. To

Figure 2 Acid-labile GlcNAc residues are present in a protein fraction enriched in acid hydrolases

A protein fraction from about 30 hepatopancreases, enriched in acid hydrolases, was degraded with a non-specific protease and the high-mannose-type glycopeptides were isolated by affinity chromatography. They were divided in half. Both halves were reduced with NaBH_4 , but one half was previously heated in 0.01 M HCl for 10 min at 100 °C. Reduced substances were successively assayed by paper chromatography in solvents A and E. In both cases substances running as a *N*-acetylglucosaminitol standard were eluted. Samples were finally assayed by paper chromatography in solvent E. For further details see the text. ○, untreated sample; ●, acid-treated sample. Standard: 1, *N*-acetylglucosaminitol.

investigate the presence of a similar enzyme in *C. granulata* a crude microsomal membrane fraction from the hepatopancreas was incubated with [β - ^{32}P]UDP-GlcNAc in the presence of α -methyl mannoside (see the Experimental section). The incubation was stopped by the addition of 1 ml of EDTA and the solution was applied to a QAE-Sephadex column. Label eluted with 30 mM NaCl was characterized. Phosphodiester (as GlcNAc α 1-*P*6Man α 1-methyl) are eluted under those conditions whereas P_i , phosphomonoesters (as GlcNAc-1-*P* or *P*6Man α 1-methyl) and diphosphates (as UDP or UDP-GlcNAc) are retained. Two labelled substances appeared when material eluted with 30 mM NaCl was assayed by paper chromatography (Figure 3a). The fast compound migrated as a GlcNAc α 1-*P*6Man α 1-methyl standard. The slow peak appeared even when α -methyl mannoside was omitted from the incubation mixture. Alkaline phosphatase treatment of the fast substance in Figure 3(a) did not modify its migration, thus indicating that the phosphate groups were covered (Figure 3b). A mild acid treatment (30 min at 100 °C in 0.01 N HCl) of the fast substance, however, produced a compound that migrated as a *P*6Man α 1-methyl standard (Figure 3c). Alkaline phosphatase treatment of the labelled substance in Figure 3(c) produced P_i , thus indicating that the mild acid treatment had uncovered those residues (Figure 3d). The labelled products shown in Figures 3(c) and 3(d) also migrated as *P*6Man α 1-methyl and P_i standards, respectively, when assayed by paper electrophoresis in solvent C (not shown).

Only one labelled substance appeared when UDP-[^3H]GlcNAc was used as sugar donor. The reaction product migrated as a GlcNAc α 1-*P*6Man α 1-methyl standard (Figure 3e). On mild acid hydrolysis it was decomposed to GlcNAc (Figure 3f).

Figure 3 Characterization of the reaction product of the UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase

C. granulata membranes were incubated with [β - ^{32}P]UDP-GlcNAc and α -methyl mannoside. Reaction products retained by a QAE-Sephadex column and eluted with 30 mM NaCl were assayed by paper chromatography (a). The fast-migrating substance in (a) was eluted and treated with alkaline phosphatase (b). The fast-migrating substance in (a) was eluted and heated for 30 min at 100 °C in 0.01 M HCl (c). The labelled substance in (c) was eluted and treated with alkaline phosphatase (d). In (e), membranes were incubated with UDP-[^3H]GlcNAc and α -methyl mannoside. Reaction products retained by a QAE-Sephadex column and eluted with 30 mM NaCl were assayed by paper chromatography (e). The labelled substance in (e) was eluted and treated with mild acid as above (f). In all cases the panels represent paper chromatography in solvent G. Standards: 1, GlcNAc α 1-*P*6Man α 1-methyl; 2, *P*6Man α 1-methyl; 3, P_i ; and 4, GlcNAc.

Results depicted in Figure 3 show, therefore, that *C. granulata* microsomal membranes have an enzymic activity that transfers GlcNAc-1-*P* from UDP-GlcNAc to α -methyl mannoside. The enzyme had an optimum pH of 7.0 and it required a bivalent cation, Mn^{2+} being more effective than Mg^{2+} (results not shown).

The enzyme phosphorylated two lysosomal enzymes (bovine uteroferrin and *C. granulata* α -L-fucosidase) whereas a non-lysosomal glycoprotein (bovine pancreatic ribonuclease B) was not phosphorylated at all (Figure 4). The enzyme detected is, therefore, a UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase.

The presence of a GlcNAc-1-phosphodiester α -*N*-acetylglucosaminidase in microsomal membranes of *C. granulata*

In order to eliminate all acid hydrolases that could be bound either non-specifically or through Man6*P* receptors to microsomal membranes, these were resuspended in 5 mM sodium maleate buffer, pH 6.8, containing 0.1 % (v/v) Triton X-100 and 10 mM Man6*P*, then sonicated and centrifuged. The precipitate was treated as above but without the addition of Man6*P* (see the Experimental section). The pellet was then incubated with [^{14}C]GlcNAc α 1-*P*6Man α 1-methyl and the incubation stopped with 2 mM Trizma and applied to a QAE-Sephadex column. Non-retained material was identified as GlcNAc by paper chromatography (Figure 5a). The reaction was time dependent (Figure 5b). The optimum pH was neutral and about 3-fold

Figure 4 Phosphorylation of glycoproteins

The indicated amounts of *C. granulata* α -L-fucosidase (○), of bovine uteroferrin (●) or of bovine pancreatic ribonuclease B (△) were incubated with microsomal membranes, and [β - 32 P]UDP-GlcNAc and the incorporation into 20% (w/v) trichloroacetic acid-insoluble material was quantified. For further details see the text.

Figure 5 Detection of a GlcNAc-1-phosphodiester α -N-acetylglucosaminidase

(a) *C. granulata* microsomal membranes were incubated with [14 C]GlcNAc α 1-P6Man α 1-methyl, and label not retained by a QAE-Sephadex column was assayed by paper chromatography in solvent G. Standard: 1, GlcNAc. (b) Membranes were incubated for the indicated times with the same substrate. For further details see the text.

more product was formed at 22 °C than at 37 °C (results not shown). These results confirm the presence of a GlcNAc-1-phosphodiester α -N-acetylglucosaminidase in microsomal membranes of the hepatopancreas.

Presence of Man6P receptors in microsomal membranes

Microsomal membranes from the hepatopancreas were freed from acid hydrolases using a procedure similar to, but not identical with, that described above (see the Experimental section). Membranes were then solubilized with 1% (v/v) Triton

Figure 6 Detection of Man6P receptors

The Man6P receptors were isolated as described in the Experimental section, precipitated with 20% (w/v) trichloroacetic acid and assayed by PAGE under denaturing conditions. Proteins were transferred to nitrocellulose papers and developed with antibodies raised against the bovine large (215 kDa) (a) or small (46 kDa) (b) Man6P receptors. The presence of the antibodies was revealed by [125 I]-labelled Protein A. Gels had 7% (w/v) (a) or 10% (w/v) (b) acrylamide. Lanes A, applied material; lanes B, material eluting with 2 mM Glc6P; lanes C, material eluting with Man6P. The molecular masses of standard proteins are indicated at the left.

X-100 plus 0.1% (w/v) sodium deoxycholate and applied to a phosphomannan-Sepharose 4B column. The phosphomannan was prepared from *H. holstii* and was treated with mild acid before coupling it to Sepharose 4B in order to expose Man6P residues. The column was washed with buffer and then with buffer containing first Glc6P and then Man6P. Applied and eluted material was assayed by PAGE under denaturing conditions, transferred to nitrocellulose membranes and revealed with polyclonal antisera raised against bovine large and small Man6P receptors. The antiserum raised against the large one (molecular mass of 215 kDa, also called cation-independent or Man6P/insulin-like growth factor II receptor) recognized two proteins of molecular masses of 205 and 215 kDa in the applied material (Figure 6a, lane A) and in that eluted with Man6P (Figure 6a, lane C) but not in that eluted with Glc6P (Figure 6a, lane B). The antiserum raised against the small Man6P receptor (molecular mass of 46 kDa, also called cation-dependent receptor) recognized a broad band in the applied material in the position of the 205–215 kDa proteins and several others of lower mass (Figure 6b, lane A). No material eluted with Glc6P was recognized by this antiserum (Figure 6b, lane B) whereas material eluted with Man6P gave a positive signal in the position of proteins having a molecular mass of 205–215 kDa (Figure 6b, lane C).

No positive signals were obtained in Figure 6 if the antisera against Man6P receptors were omitted from the detection procedure, thus indicating that the crab-derived material had no Protein A-recognizing proteins. As pre-immune sera were not available, they were not tested for the presence of immunoglobulins recognizing crab proteins. However, the possibility that positive signals observed in Figure 6 could be due to immunoglobulin binding to proteins other than Man6P receptors seems highly unlikely, as *C. granulata* membrane proteins recognized by the antisera were present not only in crude extracts but also in material retained by the phosphomannan-Sepharose 4B column and eluted with Man6P but not with Glc6P. Moreover, the size of the recognized material was similar to that of the mammalian receptor.

On the other hand, it was checked that *C. granulata* had

receptors that recognized α -L-fucosidase in a Man6P-dependent way: the crab hydrolase was labelled with Na¹²⁵I and incubated with membranes previously washed as described for experiments shown in Figure 6 for 0, 30 or 60 min at 22 °C or for 60 min at the same temperature but in the presence of 10 mM Man6P. The membranes were thoroughly washed and assayed by 10 % (w/v) PAGE under denaturing conditions. Autoradiography only showed positive signals corresponding to α -L-fucosidase in samples incubated for 30 and 60 min in the absence of Man6P.

It may be concluded that *C. granulata* microsomal membranes contain two proteins of molecular masses of 215 and 205 kDa that behave as Man6P receptors. The possibility cannot be discarded that the 205 kDa protein was produced by proteolytic degradation of the 215 kDa one during preparation of the membrane extract. No small receptor with a size comparable with that of the mammalian 46 kDa one was detected in the estuary crab. On the other hand, the fact that both antisera recognized the same proteins in the estuary crab is not surprising, as in mammals both receptors share 14–28 % identity in their cytoplasmic sequences [27].

Evidence presented here strongly suggests that the Man6P-dependent routing mechanism of lysosomal enzymes is operative in the hepatopancreas of the estuary crab *C. granulata*, because (a) an acid hydrolase was shown to contain mannose-linked phosphate residues; (b) high-mannose-type oligosaccharides isolated from a protein extract enriched in acid hydrolases were shown to contain acid-labile GlcNAc units; (c) a UDP-GlcNAc:lysosomal enzyme GlcNAc-1-phosphotransferase that phosphorylated both estuary crab and mammalian lysosomal enzymes but not non-lysosomal glycoproteins was detected in membranes of the hepatopancreas; (d) a GlcNAc-1-phosphodiester α -N-acetylglucosaminidase was present in the above-mentioned membranes; and (e) two proteins of molecular masses of 205 and 215 kDa were identified as putative Man6P receptors, as they were retained by a Man6P-rich mannan-Sepharose 4 B column from where they were eluted with Man6P but not with Glc6P. Moreover, they were recognized by antisera raised against both mammalian Man6P receptors.

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