A Lysosomal Cysteine Proteinase from Dictyostelium discoideum Contains N-Acetylglucosamine-1-phosphate Bound to Serine but Not Mannose-6-phosphate on N-linked Oligosaccharides*

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Proteinase-1 was described previously as an abundant lysosomal cysteine protease from Dictyostelium discoideum amoeba growing on bacteria (1). More recently, we found that Dictyostelium discoideum cell lysosomal cysteine proteases typically contain one or more N-linked oligosaccharides (6), and all known Dictyostelium lysosomal enzyme families have phosphorylated and sulfated N-linked chains (7). The presence of mannose-6-phosphate as a phosphomethyl disulfide (Man-6-P-OCH3)

EXPERIMENTAL PROCEDURES

Materials—PNGase-F was purified as described (14). Alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG were from Promega (Madison, WI). Nitrocellulose membranes and protein A-agarose were obtained from Schleicher & Schuell. Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, wheat germ agglutinin-agarose, concanavalin A-Sepharose, bovine serum albumin, L- and d-fucose, UDP-GlcNAc, GlcNAc-1-P, UDP-Glc, UDP-GalNAc, N-t-(N-tert-butyxycarbonyl)-Val-Leu-Lys-(7-amido-4-methylcoumarin), and goat anti-
Phosphorylase of a Cysteine Protease

Growth and development were prepared as described (2). All other chemicals were of reagent grade. Concanavalin A-Sepharose binding of proteinase-1 was performed on 2 × 0.5 cm columns equilibrated in TBS at pH 7.2. Sample was loaded in 0.5 ml of TBS and allowed to bind for 15 min. Four 1-ml fractions were collected following elution with 4 × 1-ml fractions of 100 mM α-methyl mannoside in TBS. Each fraction was assayed for proteinase-1 activity as described (1). Wheat germ agglutinin affinity chromatography was performed on a 1 × 0.5 cm column by adding the sample in 0.5 ml of TBS and allowing it to bind for 15 min. 4 × 1-ml fractions were collected followed by elution with 4 × 1 ml of 100 mM α-methyl Gal and finally by elution with 3 × 1 ml of 100 mM GlcNAc. Lysosomal enzymes were prepared from axenic cells after their secretion into non-nutrient phosphate buffer for 3 h (16).

Carbohydrate Analyses—The carbohydrate composition was determined on a sample that was hydrolyzed in 2 M trifluoroacetic acid for 4 h at 100 °C and then injected into a Carbopac PA-1 column and detected with a Dionex DX-500 system (Sunnyvale, CA) equipped with a pulsed amperometric detector (UCSD Glycobiology Core). Identification and quantitation was based on co-elution and detector response using known monosaccharide standards (17). Sugars obtained by β-elimination were directly injected on a Carbopac PA-1 column eluted for 5 min with 100 mM sodium hydroxide followed by a 60-min linear gradient to 400 mM sodium acetate. Standard Fuc, GlcNAc, and GlcNAc-α-1-P were used for calibration. Sugar alcohols were analyzed on a Carbopac MA-1 column following β-elimination or acid hydrolysis (as above) of the glycoprotein. Elution was done with a linear gradient from 20 to 500 mM sodium hydroxide over 85 min. In these conditions, FucOH, GalNAcOH, GlcNAcOH, and their de-N-acetylated derivatives are separated. Monosaccharides were analyzed after acid hydrolysis (same as above) on a Carbopac PA-1 column eluted isocratically with 16 mM sodium hydroxide. The elution position and detector response were determined with authentic standards. Composition analysis was also performed on a Finnegan GLC mass spectrometer using trimethylsilyl derivatives of the de-N-acetylated methyl glycosides and arabitol as an internal standard (18). Identity of the individual sugars was confirmed by retention time and by comparison of the mass spectra with authentic standards.

β-Elimination and Analysis of Proteinase 3—500 μg of purified proteinase 3 was analyzed against variously, lyophilized, and reduced by adding 0.5 ml of 0.05 N NaOH at 22 °C for 2 h, neutralized with 0.05 N HCl, and passed over a 0.7 × 20-cm column of Biogel P-2 equilibrated in water. Fractions were taken and aliquots were monitored at 241 nm (measures the double bond formation after β-elimination from Ser or Thr) or assayed for reducing sugar by the method of Park and Johnson (19) following acid hydrolysis. Carbohydrate positive fractions were pooled and subjected to carbohydrate analysis using a Carbopac PA-1 column as described above. Alternatively, the pooled sample was used for LSIMS as described above. Above, the determination of proteinase, a similar sample of 200 μg was analyzed following reductive β-elimination with 0.2 N NaOH at 22 °C in the presence of NaBH4 for 24 h. The released material was isolated following neutralization and passage over a mixed-bed ion-exchange resin (MB-3). Fucitol was analyzed using the pulsed amperometric detection system.

LSIMS—Liquid secondary ion mass spectrometry analysis was performed with a VG 70-5E (VG Instruments) magnetic sector mass spectrometer in the negative ion mode. The instrument was equipped with a cesium ion gun operated at 23 kV and an emission current of 4–5 mA. Spectra were recorded in the mass range 100-1000 mass to charge ratio. The sample (10 μg) dissolved in 5 μl of 40% methanol was applied to the stainless steel target holding 2 μl of glycerol/thiodiglycerol matrix, and 1 μl of 1 N HCl was added. The scans were repeated after the addition of sodium chloride to confirm the identity of the molecular ion. An authentic standard was analyzed using the same conditions.

Immunological Procedures—Polyclonal antibodies against proteinase-1 were prepared as described (1). IgG fraction was prepared by passing the serum over a protein A-agarose column (Schleicher & Schuell) according to the manufacturer’s directions and eluted at pH 2.5. All active antibodies bound to the column. Affinity purification of antibodies against GlcNAc-α-1-P was on a UDP-GlcNAc column as described previously (20).

Production of Monoclonal Antibodies—Four-week-old BALB/c mice were injected intraperitoneally with 100 μg of purified proteinase-1 in 1 ml of PBS. Three subsequent boosters were given at 3-week intervals with 35–50 μg of proteinase-1 in incomplete Freund’s adjuvant. Mice were given 50 μg of proteinase-1 in phosphate-buffered saline 3 days prior to the fusion. Spleen cells were fused with P3 × 63-Ag8.653 myeloma cells at 2:1 ratio in the presence of 42% polyethylene glycol in serum-free medium. Growth and maintenance of hybridomas were performed as described (21). IgG was purified from ascites fluid using the caprylic acid method (22). Mouse isotyping kits (Bio-Rad Laboratories) was used to determine the IgG subclass.

ELISA Assays—Titer of the antiserum, screening of hybridoma supernatants, and inhibition of antibody binding to proteinase-1 were measured by an ELISA assay. Proteinase-1 was dried at 50 °C for 1 h at the bottom of a plastic 96-well plate. Blocking was done with 3% bovine serum albumin in TBS for 1 h. An appropriate dilution of the antibody was added and incubated for 1 h in the presence or the absence of the indicated amount of inhibitor. The plate was washed and incubated with a 1:1000 dilution of alkaline phosphatase-conjugated secondary antibody. After washing with TBS containing 0.05% Tween-80, the plates were incubated with 1 mg 4-methylumbelliferyl phosphate for 30–60 min and diluted to 3 ml, and the fluorescence was read on a Turner fluorometer (23). When 3 mg p-nitrophenyl phosphate was used as the substrate, the resultant color was read on an ELISA plate reader.

Immunoprecipitation—Vegetative Dictyostelium cell extracts were made as lysing cells (grown on bacteria and harvested on clearing) with buffer A (0.1 M Tris·HCl, pH 7.5, containing 150 mM NaCl, 0.1% Nonidet P-40, and 1 mM dithiothreitol) and then precladred with Proteinase-1 for 2 h at 4 °C. 10 μg of precladred proteins were mixed with different aliquots of purified mAb AD7.5 and incubated overnight with rocking at 4 °C. 2-fold excess of goat anti-mouse IgG over the mAb was then added and further incubated at 4 °C for 2 h. Proteinase-1 was added in excess to pellet all the antibodies and allowed to incubate for 2 h at 4 °C. The pellet was collected by centrifugation at 10,000 × g for 5 min and washed 3–4 times with buffer A. It was then used either for proteinase assay using N-t-(N-tert-butoxycarbonyl)-Val-Leu-Lys-(7-amido-4-methylcoumarin) (2) or for the lysosomal enzymes. Specificity was determined by incubations with 1 μg UDP-GlcNAc. Immunoprecipitation with CI-M6PR and anti-CI-M6PR was done with Proteinase-1 precladred extract prepared as above. Varying amount of purified CI-M6PR was added to 0.5 μg of precladred extract and incubated for 5–6 h at 4 °C followed by the addition of Proteinase-1 coated with anti-M6PR and further incubated overnight at 4 °C. Specific precipitation was determined by ELISA competition with 5 μg Man-6-P.

Western Blots—Mini 12.5% SDS-gels were blotted onto nitrocellulose paper and blocked with 5% powdered milk TBS containing 0.05% Tween-80 (25). After washing in TBS containing 0.05% Tween-80, the blots were incubated for 1 h with appropriate dilutions of antibodies. Blots were washed and incubated for 1 h in alkaline phosphate-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (dilution 1:1000) prior to developing with 5-bromo-4-choloro-3-indolyl phosphate and nitro blue tetrazolium (25). Man-6-P containing oligosaccharides on blotted proteins were detected by incubation with 1 μg/ml bovine CI-MPR in TBS containing 0.05% Tween-80, followed by rabbit antiserum against CI-MPR (1:1000 dilution) and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG. In control experiments, nor- over, did we observe any cross-reactivity with 5 μg Man-5-P.

Enzyme Digestions—PNGaseF digestions were performed on heat and SDS denatured samples of proteinase-1 using 1 milliunit of enzyme in a total volume of 50 μl at pH 7.0 as described previously (26). After digestion, the protein was analyzed by Western blotting.

RESULTS

Carbohydrate Composition and Preliminary Carbohydrate Chain Characterization—Previous studies showed that proteinase-1 contains only GlcNAc-α-1 linked to serine, and it accounted for nearly 20% of the mass of the protein (4, 5). No other sugars were reported. We purified proteinase-1 using an established method and found that it bound to a wheat germ
agglutinin-agarose column and was eluted by 100 mM GlcNAc. Because Dictyostelium does not synthesize sialic acids (35), this supported the presence of clustered GlcNAc residues in the protein that is consistent with the presence of GlcNAc-α-1-P. We re-examined the carbohydrate composition of purified proteinase-1 by gas chromatography/mass spectrometry analysis of the trimethylsilylated methyl glycosides and by direct high performance anion exchange chromatography-pulsed amperometric detection analysis as described under “Experimental Procedures.” The results obtained by two methods agreed well, and the average compositions are shown in Table I. In contrast to previous studies (4, 5), these analyses showed the presence of fucose and mannose and a small amount of xylose. Variable amount of glucose is most likely a contaminant. Significantly, these analyses showed no evidence for mannose-6-phosphate, which is normally found on N-linked chains of Dictyostelium lysosomal enzymes. To determine whether the various sugars occurred on N- and O-linked chains, the carbohydrate composition was determined before and after digestion with PNGase F to release N-linked oligosaccharides. The released chains were isolated by gel permeation chromatography, and their composition was determined. Table I shows that the majority of the mannose and xylose was sensitive to PNGase F, whereas less than 10% of the fucose and GlcNAc were released.

Identification of GlcNAc-α-1-P in Proteinase-1—To determine the type of linkage involved in GlcNAc and fucose, purified proteinase-1 was treated with 0.05N NaOH at 22 °C for varying times. This treatment caused a dramatic increase in absorbance at 241 nm, which reached a maximum by 1 h (not shown). This change is due to the formation of an unsaturated bond in the linkage amino acid during β-elimination of the carbohydrate moiety (27). The sample was neutralized and fractionated on a Biogel P-4 column. The void region containing the protein was pooled separately from the released material. The latter was analyzed by LSIMS (mass accuracy of ±0.5 mass unit) as described under “Experimental Procedures.” As shown in Fig. 1, a major molecular ion was detected at m/z 300. The pattern observed corresponded exactly to that obtained for the GlcNAc-α-1-P standard. The predicted theoretical mass of GlcNAc-α-1-P was 299.17 and the observed [M+H] value was 300, which is within the error of the instrument. No peaks were detected beyond m/z 500, indicating the absence of larger structures. Complete acid hydrolysis of the sample followed by high pressure liquid chromatography analysis showed only GlcNH2 in this fraction. Because no fucose was released, β-elimination was repeated using harsher conditions of 0.2 N NaOH for 24 h at 22 °C in the presence of NaBH4. This is sufficient to release O-linked fucose from mammalian glycoproteins as fucitol (28), but HPAEC-PAD in a CarboPac PA-1 column showed the presence of only GlcNAc-α-1-P and no evidence of fucitol. Taken together, these results show that all of the base released GlcNAc occurs as GlcNAc-α-1-P. However, the linkage of fucose to the protein or to other sugars remains unresolved. Fucose is not likely to be retained in a PNGaseF-resistant N-linked oligosaccharide, because nearly all mannose was released by digestion with this enzyme.

Preparation and Characterization of Rabbit Antibodies against Proteinase-1—We prepared an antiserum against proteinase-1 and purified the IgG fraction by repeated passage over a column of immobilized UDP-GlcNAc to select for antibodies that recognize GlcNAc-α-1-P (1, 19). The affinity purified antibody bound to proteinase-1 and was 50% inhibited by 0.08 mM UDP-GlcNAc, 0.1 mM GlcNAc-α-1-P, 0.7 mM UDP-GalNAc, and >1 mM UDP-Glc. No inhibition was seen with GaINac, Glc, Man, Gal-1-P, Man-1-P, Glc-1-P, UDP-Gal, or serine phosphate at 0.5 mM. On Western blots with proteinase-1, these antibodies recognize a major band at 38 kDa and two faint bands at 36 and 55 kDa. Binding to the three bands was competed by 12.5 mM UDP-GlcNAc (Fig. 2A).

Further Analysis of N-linked Chains—Proteinase-1 bound to concanavalin-Sephrose and was eluted with α-methyl mannoside (not shown). As mentioned above, digestion of proteinase-1 with PNGase F releases a portion of the carbohydrate and reduces the apparent size of the major band by approximately 1.5 kDa (Fig. 3), but the binding of GlcNAc-α-1-P specific rabbit antibody is not affected. This is consistent with the results obtained by composition analysis of the PNGase F released material and suggests the presence of probably a single N-linked chain. However, as expected, proteinase-1 still retains full reactivity with the affinity purified GlcNAc-α-1-P-specific antibody. In contrast, PNGase F digestion greatly reduces the binding of monoclonal antibody mLE2 directed against common antigen 1, a group of mannose-6-sulfate residues on N-linked oligosaccharides of Dictyostelium lysosomal enzymes (12). These sugar chains typically also contain Man-6-P-OCH3 residues that mediate their binding to the mammalian CI-MPR (8–11). As shown in Fig. 3, immunoblots with the CI-MPR showed no binding of purified proteinase-1 but reacted strongly with a similar amount of a mixture of Dictyostelium lysosomal enzymes with phosphorylated oligosaccharides. The binding to CI-MPR is lost if the proteins are first treated with PNGase F (Fig. 3) or if binding is carried out in the presence of 5 mM Man-6-P (not shown). These results further confirm the direct carbohydrate compositional analysis, showing that proteinase-1 does not contain Man-6-P, which makes proteinase-1 quite unusual compared with other lysosomal enzymes in Dictyostelium.

Presence of Fucose on Non-N-linked Structures—Monoclonal antibody 83.5 recognizes α-fucose residues in an unknown linkage to several proteins in Dictyostelium (29). As shown in Fig. 3, proteinase-1 also contains the epitope recognized by mAb 83.5 in a PNGase-F-resistant linkage that is competed with

| Sugar | Native | PNGase-F-sensitive | PNGase-F-resistant |
|-------|--------|--------------------|-------------------|
| GlcNAc | 35.1 | 3.0 | 29.0 |
| Fucose | 6.3 | 0.3 | 5.5 |
| Mannose | 5.7 | 3.8 | 0.4 |
| Xylose | 1.9 | 1.0 | 0.4 |

*a Calculated from molecular mass of 38 kDa determined by SDS-polyacrylamide gel electrophoresis and protein content estimated by Folin's method.

**TABLE I**

Carbohydrate composition of proteinase-1

| Sugar | Protein* |
|-------|----------|
| GlcNAc | 30.0 |
| Fucose | 7.5 |
| Mannose | 8.0 |
| Mannose-6-P | 0.0 |
| Xylose | 2.0 |

*Calculated from molecular mass of 38 kDa determined by SDS-polyacrylamide gel electrophoresis and protein content estimated by Folin's method.
L-fucose but not D-fucose (Fig. 2B). This result confirms that fucose occurs on proteinase-1 and is not a contaminant in the preparation. Digestions with almond α-L-fucosidase, which cleaves α(1–3,4) linked residues (30), or chicken liver α-L-fucosidase, which cleaves α(1–2,4,6) linkages (31), could not remove these fucose residues. We have been unable to identify how fucose residues are linked to the protein.

Monoclonal Antibody Recognizing GlcNAcα-1-P—Monoclonal antibodies against GlcNAcα-1-P were made in mice immunized with proteinase-1. 26 positive hybridomas bound to purified proteinase-1 by ELISA screening, and three of these were specifically inhibited with UDP-GlcNAc or GlcNAcα-1-P. One of these, AD7.5, was used for further studies, and as shown in Fig. 4, its binding to proteinase-1 is inhibited best by UDP-GlcNAc at amounts comparable with that required for inhibition of the affinity-purified GlcNAcα-1-P-specific rabbit antibody. AD7.5 was also inhibited with UDP-Glc and GlcNAcα-1-P and partially with UDP-GalNAc but was unaffected by 5 mM Glc-6-P, GlcNAc, GalNAc, and serine phosphate. The three GlcNAcα-1-P-specific antibodies recognized the protein on Western blots, suggesting that most of the other antibodies recognize peptide portions of native proteinase-1. mAb AD7.5 recognized the same three bands on Western blots as seen by the polyclonal antibody, and this reactivity could be competed with 10 mM UDP-GlcNAc (not shown).

mAb AD7.5 recognizes other proteins during various times of growth and development, but densitometer scans showed that more than 90% of the GlcNAcα-1-P reactivity is found at the end of vegetative growth and proteinase-1 is the major carrier of GlcNAcα-1-P (Fig. 5). However, other cysteine proteinases in Dictyostelium are also known to have this modification (2, 3).

Reactivity of mAb AD7.5 with Other Lysosomal Enzymes—Because proteinase-1 did not contain Man-6-P, we wanted to determine whether other lysosomal enzymes with phosphorylated oligosaccharides also had GlcNAcα-1-P. To do this, monoclonal antibodies against α-mannosidase (32), β-glucosidase (33), and acid phosphatase (34) were used to precipitate the
activities from extracts of cells growing in liquid medium. The precipitates were then solubilized and analyzed in immuno-
blots using CI-M6PR and anti-M6PR or polyclonal GlcNAc-
1-P antibody. Each enzyme bound CI-M6PR and its antibody, but none reacted with the GlcNAc-
1-P antibody (not shown).

To expand this finding, cell extracts made from bacterially grown Dictyostelium were mixed with either increasing amounts of CI-M6PR and anti-CI-M6PR or mAb AD7.5. As shown in Fig. 6, mAb AD7.5 completely precipitated all of the cysteine proteinase activity, which includes proteinase-1 and at least two other cysteine proteinases (3). However, it did not precipitate any acid phosphatase, β-glucosidase, or α-glucosidase activities. Technical difficulties prevented a similar analysis of α-mannosidase in these samples. Instead, a partially purified preparation of α-mannosidase was analyzed. Increasing amounts of mAb AD7.5 that completely precipitated an equal amount of purified proteinase-1 did not precipitate any α-mannosidase activity (Fig. 6B). Conversely, CI-M6PR and anti-CI-M6PR precipitated 30–70% of the typical lysosomal enzymes containing Man-6-P, including α-mannosidase, α-glucosidase, and β-glucosidase, but none of the cysteine proteinase activity (Fig. 6C). These results show that a representative selection of lysosomal enzymes have either GlcNAc-1-P or Man-6-P but not both modifications on the same protein.

An exception to this pattern was NAG, which had been previously shown to react with the affinity purified GlcNAc-1-P-specific rabbit antiserum (13). In crude extract, we found that about 50% of NAG activity was precipitated with AD7.5. However, 95% of NAG activity was lost during the preclearing step, prior to the addition of antibody. This large loss of activity could mean that the sample we analyzed was not a representative. To determine whether NAG also contains GlcNAc-1-P, we used a preparation of lysosomal enzymes that was highly enriched in NAG and β-glucosidase activities and also contained α-glucosidase and proteinase-1 activity. Fig. 7 shows that mAb AD7.5 completely precipitates cysteine proteinase activity, whereas it precipitates only 15% of NAG, none of the β-glucosidase, and less than 3% of α-glucosidase activities. All of the precipitations were inhibited with 1 mM UDP-GlcNAc. The reverse pattern is seen with CI-M6PR and anti-CI-M6PR.
tion analysis and LSIMS of this sugar released by
also contained the GlcNAc-
minewhether the major isozyme that is known to have Man-6-P
portion of NAG was immunoprecipitated, we wanted to deter-
minor form is the most stable at 55°C (35). Because only a minor
and has a half-life of 15–17 min at 55°C (35), whereas the
cysteine proteinase activity.

It precipitates 40–90% of the glycosidases, but none of the
cysteine proteinase activity.

NAG activity is coded by two different genes (35). The major
form in axenically growing cells accounts for 90% of the activity
and has a half-life of 15–17 min at 55 °C (35), whereas the
minor form is thermostable at 55 °C (35). Because only a minor
portion of NAG was immunoprecipitated, we wanted to deter-
mine whether the major isozyme that is known to have Man-6-P
also contained the GlcNAc-1-P epitope. More than 85% of the
partially purified GlcNAc was thermostable at 55 °C (t1/2 = 12
min) and is therefore the major isozyme (data not shown). The
thermostable activity (~15%) is probably the minor
isozyme. After inactivating the major enzyme at 55 °C, mAb
AD7.5 still only immunoprecipitated 10% of the remaining
NAG activity. If GlcNAc-1-P occurred only on the minor
isozyme, the proportion of the thermostable precipitable activity
should have increased. Because it did not, this suggests that
a small fraction of the major isozyme contains GlcNAc-α-1-P.
The 10% of thermostable activity that is precipitated could
represent the residual activity of the major form.

DISCUSSION

Proteinase-1 is rich in GlcNAc-α-1-P as shown by composi-
tional analysis and LSIMS of this sugar released by β-elimina-
tion (Table I and Fig. 1). This agreed with previous studies by
others (4, 5). In contrast to earlier reports, we also found
evidence for the presence of N-linked oligosaccharides based on
compositional analysis, lectin binding, and PNGaseF sensitiv-
ity, which released the great majority of the mannose and some of
GlcNAc (Tables I and II). These results clearly show that
GlcNAc-α-1-P is not a part of N-linked chains. Fucose residues
were also detected in hydrolysates of proteinase-1 (Table I), and
a fucose-specific monoclonal antibody confirmed that it
was bound to the protein (Figs. 2B and 3). However, these
residues were not found on N-linked chains (Table II), nor did
they appear to be bound to GlcNAc-α-1-P residues. They were
not released by any α-fucosidase digestion or as fucitol follow-
base treatment (Fig. 1). Thus, although fucose is clearly
present, it may be linked to the protein via another base-
resistant linkage.

The N-linked chains of proteinase-1 contain a modification
that is recognized by a series of monoclonal antibodies that
bind to a cluster of mannose-6-SO4 residues found in Dictyo-
stelium (12). This modification is found almost invariably along
with Man-6-P-OCH3 in the previously analyzed Dictyostelium
lysosomal enzymes (8, 9) and is analogous to that seen in
mammalian cells. It accounts for the binding of Dictyostelium
lysosomal enzymes to the mammalian CI-MPR (10, 11). How-
ever, direct analysis of acid hydrolysates showed no mannose-
6-P residues (Table I), and immunoblots using the CI-MPR
showed no reactivity against proteinase-1 (Fig. 3). This was
highly unusual for a Dictyostelium lysosomal enzyme. Presence
of xylose is known to occur in Dictyostelium proteins (36) and is
usually in a β 1–2 linkage to the mannose of an N-linked chain.
Although we have not analyzed this in detail, it is tempting to
speculate that xylose could be in a similar linkage.

The GlcNAc-α-1-P-specific monoclonal antibody showed that
an entire family of at least 3–4 co-regulated proteins all con-
tain this modification (3) (Fig. 5). The major bands recognized
by this antibody show cysteine proteinase activity (2, 3) and are
the major carriers of this modification in Dictyostelium. Al-
though most cysteine proteinases are well conserved in eukary-
totes, at least two members of this cysteine proteinase
family in Dictyostelium have an unusual serine-rich domain
that probably accommodates the GlcNAc-α-1-P residues (3, 37).
However, none of these proteinases contain Man-6-P residues
(Figs. 6 and 7). This contrasts with α-mannosidase and α and
β-glucosidases that have been shown to contain Man-6-P (7–9)
but do not have GlcNAc-α-1-P (Fig. 6). An apparent exception
to this pattern is seen with NAG, because it is known to contain
Man-6-P but was also precipitated with the GlcNAc-α-1-P
antibody (Fig. 7). Earlier studies with polyclonal serum against
proteinase-1 also showed cross-reactivity with NAG (13). One
possible explanation is that the reactivity is due to a contami-
nation with a minor NAG isozyme. However, differential
thermolability of the two forms showed that the minor enzyme
could not account for the GlcNAc-α-1-P reactivity and implied
that only a small portion of the major form is modified by
GlcNAc-α-1-P.

We have not examined all lysosomal enzymes for the pres-
ence of GlcNAc-α-1-P and Man-6-P, but our initial results sug-
gest that they occur on different types of lysosomal enzymes.
There is no information on how the two types of enzymes are
distinguished from each other, but two other Dictyostelium
cysteine proteinases recently cloned by our lab (3) and protein-
ase-1 (2) all contain serine-rich regions that could be sites of
phosphoglycosylation. α-Mannosidase also has a serine-rich
region, but it clearly does not contain GlcNAc-α-1-P (38). A
peptide containing a series of Ser-Gly repeats serves as a good
acceptor (Km = 200 μM) for GlcNAc-α-1-P transferase (37),
showing that the intact protein is not required for recognition.
This transferase is clearly distinct from GlcNAc-α-1-P transfer-
ase catalyzing the first step in biosynthesis of Man-6-P on N-
linked oligosaccharides (39).
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The mechanism of lysosomal enzyme targeting in Dictyostelium has not been resolved, especially in regard to the role of Man-6-P-OCH3. Clearly, if it can be used for targeting, the Man-6-P containing enzymes are highly expressed (40), whereas the cysteine proteinase activity is lower in axenic medium (2, 3, 41). The large increase in cysteine proteinase activity at the onset of development may be linked to some specialized function at the growth-development transition to their unusual carbohydrate modification or to the decrease in activity at the onset of development would not be expected if these enzymes are being used only for digesting bacteria. The rapid increase in activity at the onset of development may be linked to some specialized function at the growth-development transition to their unusual carbohydrate modification or to the protein domain that carries these modifications. Preliminary studies in our laboratory suggest that the two carbohydrate modifications have distinct roles during phagocytosis of bacteria.

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