

***Dictyostelium* lysosomal proteins with different sugar modifications sort to functionally distinct compartments**

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This paper is dedicated to the memory of Sophie North

SUMMARY

Many *Dictyostelium* lysosomal enzymes contain mannose-6-phosphate (Man-6-P) in their N-linked oligosaccharide chains. We have now characterized a new group of lysosomal proteins that contain N-acetylglucosamine-1-phosphate (GlcNAc-1-P) linked to serine residues. GlcNAc-1-P-containing proteins, which include papain-like cysteine proteinases, cofractionate with the lysosomal markers and are in functional vesicles of the endosomal/lysosomal pathway. Immunoblots probed with reagents specific for each carbohydrate modification indicate that the lysosomal proteins are modified either by Man-6-P or GlcNAc-1-P, but not by both. Confocal microscopy shows that the two sets of proteins reside in physically and functionally distinct

compartments. Vesicles with GlcNAc-1-P fuse with nascent bacteria-loaded phagosomes less than 3 minutes after ingestion, while those with Man-6-P do not participate in bacterial digestion until about 15 minutes after phagocytosis. Even though both types of vesicles fuse with phagosomes, GlcNAc-1-P- and Man-6-P-bearing proteins rarely colocalize. Since both lysosomal enzymes and their bound carbohydrate modifications are stable in lysosomes, a targeting or retrieval mechanism based on these carbohydrate modifications probably establishes and/or maintains segregation.

Key words: Lysosome, *Dictyostelium*, Glycoprotein

INTRODUCTION

Lysosomal enzymes in *Dictyostelium discoideum* have been well characterized in axenic cells (Cardelli, 1993). Studies on developmental regulation, lysosomal enzyme secretion and post-translational modifications have been done with normal and mutant strains altered in these features (Cardelli, 1993). Lysosomal enzymes such as α -mannosidase and β -glucosidase have N-linked oligosaccharides containing Man-6-P in a stable phosphomethyl diester linkage (Man-6-P-OCH₃) (Freeze, 1997; Freeze and Wolgast, 1986; Gabel et al., 1984) that allows them to bind to the mammalian cation-independent Man-6-P receptor (CI-MPR) (Freeze et al., 1980). In addition, many of these same oligosaccharide chains contain a cluster of mannose-6-sulfate (Man-6-S) residues called common antigen 1 (CA1) (Freeze et al., 1990) that is recognized by a monoclonal antibody, mLE2 (Knecht et al., 1984).

In nature, amoebae feed on bacteria, and lysosomal proteases including cysteine proteinases (CPs) of the papain family play a major role in their degradation (North and Cotter, 1991; Mehta et al., 1995). Expression of different CP activities depends on the stage of growth and the food (North et al., 1996). For instance, a major CP, proteinase-1, is expressed in cells growing on bacteria and not in cells grown in axenic medium (Souza et al., 1995). Although CPs are well conserved

in eukaryotes, several cloned vegetative *Dictyostelium* CPs contain a novel serine-rich domain (Souza et al., 1995) that appears to be the site for phosphoglycosylation (Freeze and Ichikawa, 1995), the addition of phosphodiester-linked GlcNAc-1-P residues to serine (Gustafson and Gander, 1984). Deletion of this serine-rich domain from proteinase-1 abolishes recognition by a specific monoclonal antibody (AD7.5) that recognizes the sugar phosphate, suggesting that GlcNAc-1-P is probably added to this domain (Ord et al., 1997).

Proteinase-1 was the first phosphoglycosylated protein described in *Dictyostelium* growing on bacteria (Gustafson and Milner, 1980). Recently, we found that this enzyme also has N-linked oligosaccharides, but they lacked Man-6-P-OCH₃ (Mehta et al., 1996). Typical Man-6-P-OCH₃-containing lysosomal enzymes like α -mannosidase and β -glucosidase bind to the mammalian CI-MPR, but proteinase-1 does not. Conversely, a GlcNAc-1-P-specific monoclonal antibody (mAb) AD7.5 immunoprecipitates all the CP activity from bacterially grown cells, but it does not precipitate the Man-6-P-OCH₃-containing enzymes, suggesting that the two sugar modifications are on non-overlapping proteins in cells feeding on bacteria (Mehta et al., 1996). Since all of the extensive work on *Dictyostelium* lysosomal enzymes has used cells grown under axenic conditions, we wanted to study CP and GlcNAc-1-P expression under these conditions in order to facilitate

direct comparisons. We found that these two carbohydrate modifications are mutually exclusive. GlcNAc-1-P and Man-6-P-OCH₃ define different compartments of the endosomal/lysosomal (endo-lysosomal) pathway that function coordinately during phagocytosis of bacteria. Vesicles with GlcNAc-1-P-modified proteins fuse with phagosomes first, and this is followed by fusion of vesicles with Man-6-P-OCH₃-containing hydrolases.

MATERIALS AND METHODS

Materials

The molecular mass standards were from Bio-Rad Laboratories, Inc. (Hercules, CA). Alkaline-phosphatase conjugated IgGs were from Promega (Madison, WI). Percoll density gradient marker beads were from Pharmacia Biotech (Uppsala, Sweden). FITC-dextran (M_r 70,000) was from Molecular Probes, Inc. (Eugene, OR). mAb AD7.5 (Mehta et al., 1996), mAb mLE2 (Knecht et al., 1984) and mAb 6H10 were prepared as described (Mierendorf and Dimond, 1983). All other reagents were from Sigma Chemical Co. (St Louis, MO).

Cell culture

Dictyostelium discoideum strains AX-2 or AX-4 were grown axenically in HL-5 media or in association with *Klebsiella aerogenes* (vegetative cells) on SM agar plates as described (Sussman, 1987).

Phagocytosis assay

Bacteria labeled with FITC were prepared as described (Vogel, 1987). Axenically grown AX-2 cells were washed, resuspended at 5×10^6 cells/ml in 20 mM phosphate buffer, pH 6.4, and either FITC-labeled bacteria, unlabeled bacteria, FITC-latex beads (0.9 μ m) or FITC-dextran were added at a ratio of 200:1 amoeba. Cells were shaken at 200 rpm for 3 or 15 minutes (pulse), washed three times and resuspended in phosphate buffer for chasing. At the various time points samples were taken, diluted, washed and plated for confocal immunofluorescence microscopy.

Confocal immunofluorescence microscopy

Axenically or bacterially grown AX-2 cells were harvested and after extensive washing, 2.5×10^6 cells were adhered on coverslips coated with 2 mg/ml poly(lysine) on ice. Cells were fixed in 3.5% *p*-formaldehyde/PBS (phosphate-buffered saline, pH 7.2) for 1 hour, washed twice in PBS and once in 50 mM NH₄Cl, and blocked overnight in 0.2% gelatin/0.2% sodium azide/PBS at 4°C. Cells were permeabilized with 0.1% saponin/0.2% gelatin/PBS for 30 minutes followed by addition of the antibodies or CI-MPR for 1 hour. Incubations with each antibody or CI-MPR were done separately, with extensive washing with PBS after each step. CI-MPR was used at 5 μ g/ml and all antibodies were appropriately diluted in 0.3% BSA/0.1% saponin/PBS. The coverslips were mounted in 85% glycerol, pH 9, 0.02% *p*-phenylenediamine and observed on a Carl Zeiss Laser Scanning Microscope. Sections were taken every 1 μ m and the images shown are the calculated projections of all sections. The pictures shown are representative of over 100 cells analyzed. The size and number of vesicles were averaged from analysis of at least 20 pictures from five independent experiments.

Purification of lysosomes by magnetic fractionation

Vesicles of the endo-lysosome pathway were labeled by incubating cells in HL-5 for 2 hours with iron-dextran and then purified by magnetic fractionation (Rodriguez-Paris et al., 1993). Lysosomal enzyme enrichment was determined by specific activity measurements of the postnuclear supernatant (PNS), after correcting for vesicle breakage. Lysosomes were also prepared after a 15-minute pulse with colloidal iron magnetic particles followed by a 15-minute chase in the

absence of the probe. The whole lysosomes thus obtained were lysed and the membrane fraction treated with 3,5-diiodo-2-hydroxybenzoic acid lithium salt (LIS) to remove peripheral membrane proteins, as described (Temesvari et al., 1994). The supernatant from the initial membrane preparation was lyophilized and defined as lysosomal contents.

Cell fractionation by Percoll gradients

Percoll density gradient fractionation of axenically grown AX-2 cells on 24% Percoll was performed essentially as described (Bush and Cardelli, 1989; Mehta et al., 1995). CP activity and the lysosomal enzymes were assayed using their respective 4-methyl-umbelliferyl substrates (Freeze et al., 1980; Mehta et al., 1995). Density marker beads were used to monitor the density during fractionation.

Affinity purification of CI-MPR and the production of CI-MPR anti-serum

The CI-MPR was affinity purified from expired lots of fetal bovine serum as described (Sahagian et al., 1982) using a core-mannan matrix (Bretthauer et al., 1973; Cuatrecasas, 1970). The anti-CI-MPR was raised in rabbits following the procedure of Sahagian et al. (1982) and the IgG fraction was enriched as described (McKinnley and Parkinson, 1987).

Western blot analysis

Protein samples from magnetic beads and subcellular fractionation were separated on 10 or 12.5% SDS-PAGE, transferred onto nitrocellulose, incubated with antibodies and developed as described (Harlow and Lane, 1988). Quantitative determination of the amounts of Man-6-S and GlcNAc-1-P on glycoproteins was done by densitometry scans of blots developed within a linear range. Goat anti-mouse or anti-rabbit IgG alkaline phosphatase-conjugates were used for detection.

Estimation of GlcNAc-1-P-containing proteins by ELISA

ELISA was performed with 20–40 ng of protein coated in 0.1 M sodium bicarbonate and developed with AD7.5 as described (Harlow and Lane, 1988). Color was developed by the addition of 3 mM *p*-nitrophenylphosphate and quantitated at 405 nm.

Detection of GlcNAc-1-P on CPs eluted from polyacrylamide gels

30–50 μ g samples of magnetically purified lysosome extract from axenic cells were separated on 12.5% polyacrylamide gels in SDS-sample buffer without heating to preserve proteolytic activity. The gels were analyzed for CP activity using a fluorescent tripeptide substrate as described (Mehta et al., 1995). Bands of activity were excised, homogenized and eluted overnight in 0.1 M Tris-HCl, pH 7.5, at 4°C. Recovery of activity from the bands was 100–120% of the total activity loaded when assayed in solution with the same peptide substrate. Each of the eluates was concentrated with microcon-3 (Amicon) and after heating was subjected to a second SDS/PAGE and analyzed using AD7.5.

Immunoprecipitation of axenic cell extract with mAbs AD7.5 and mLE2

Cells growing at 2×10^6 cells/ml were harvested and lysed by brief sonication in TTBS (150 mM NaCl, 50 mM Tris, 0.05% Triton X-100) and centrifuged at 200 *g* for 5 minutes. The supernatant was incubated with either mAb AD7.5 or mLE2 for 2 hours at 4°C, followed by another 2 hours incubation with Protein A-Sepharose 4B beads coated with goat anti-mouse IgG. The beads were washed three times with TTBS, resuspended in SDS-sample buffer and analyzed by western blots. The blots were probed with AD7.5 or mLE2 and detected with goat anti-mouse alkaline phosphatase conjugates. 20 μ g of axenic cell extract was incubated with increasing amounts of AD7.5 and the pellets were assayed for CP activity as detailed (Mehta et al.,

1996). Samples from this precipitation were also analyzed by western blot and probed with AD7.5 to see whether the proteins precipitated with this antibody.

Stability of lysosomal enzymes and their sugar modifications

10 μg of purified proteinase-1 was incubated in the presence of 1 mM DTT at pH 4.5 and pH 6.0 at 22°C for up to 30 hours. Samples were withdrawn at different times for determination of residual enzymatic activity or analyzed using western blots with AD7.5. To verify the stability of GlcNAc-1-P to endo-lysosomal hydrolases, [^3H]GlcNAc-1-P on an *N*-octanoylated synthetic peptide (C₈GSGSGSGS) was incubated with 3-5 mg/ml of lysosomal (Percoll fraction 1) or endosomal (Percoll fraction 11) fraction at pH 5.0 or 7.0 for up to 48 hours. After heat inactivation, the samples were analyzed on C18 spin columns (Etchison et al., 1995). The column was washed with 0.1 M ammonium formate, pH 6.0, to elute unbound radioactivity resulting from hydrolysis and then with 50% methanol to elute the undegraded peptide.

RESULTS

Subcellular fractionation of CPs and phosphoglycosylated proteins

Proteinase-1 is a lysosomal CP and the major carrier of GlcNAc-1-P in bacterially grown cells (Mehta et al., 1995, 1996). Although this enzyme is not expressed in cells growing on axenic medium, such cells have additional uncharacterized phosphoglycosylated proteins, which may include other CPs. To determine whether these proteins also have a lysosomal distribution, post-nuclear supernatants (PNS) were prepared from axenically grown cells and fractionated on a Percoll density gradient. The distribution of a typical lysosomal enzyme, acid α -mannosidase, is shown in Fig. 1C. Other well-established lysosomal enzymes such as β -glucosidase, β -*N*-acetylglucosaminidase, acid α -glucosidase and acid phosphatase, all have a nearly identical distribution to that of α -mannosidase (not shown). About 47% of the α -mannosidase activity is found in dense lysosomes (fractions 1-6) and 26% in fractions 9-13, which contain markers for Golgi (Ser:GlcNAc phosphotransferase, not shown), endoplasmic reticulum (Fig. 1D, α -glucosidase-II) and endosomes (Ebert et al., 1989). As with the other lysosomal enzymes, the majority (75%) of CP activity co-fractionates in the dense lysosome region (Fig. 1B, fractions 1-6) and 16% in the endosome region (Fig. 1B, fractions 9-13), comparable to results seen in cells grown on bacteria (Mehta et al., 1995).

Quantitative immunoassays with AD7.5 show that 43% of GlcNAc-1-P reactivity codistributes with the lysosomes (Fig. 1B) and 27% is found in fractions 9-13, indicating that GlcNAc-1-P-containing proteins also distribute in endo-lysosomal compartments. mAb AD7.5 recognizes similar proteins in each Percoll gradient fraction (Fig. 1A, lane 1, fraction 1). Fractions 9-13 showed an increase in a band of 41 kDa (Fig. 1A, lane 2, fraction 11). The antibody staining was specific since the primary antibody with 10 mM UDP-GlcNAc eliminates binding (not shown). The fractions were also analyzed for Man-6-S on *N*-linked oligosaccharides of lysosomal enzymes using a monoclonal antibody (mLE2) (Knecht et al., 1984). This modification usually occurs on the same sugar chains as Man-6-P-OCH₃ (Freeze et al., 1983,

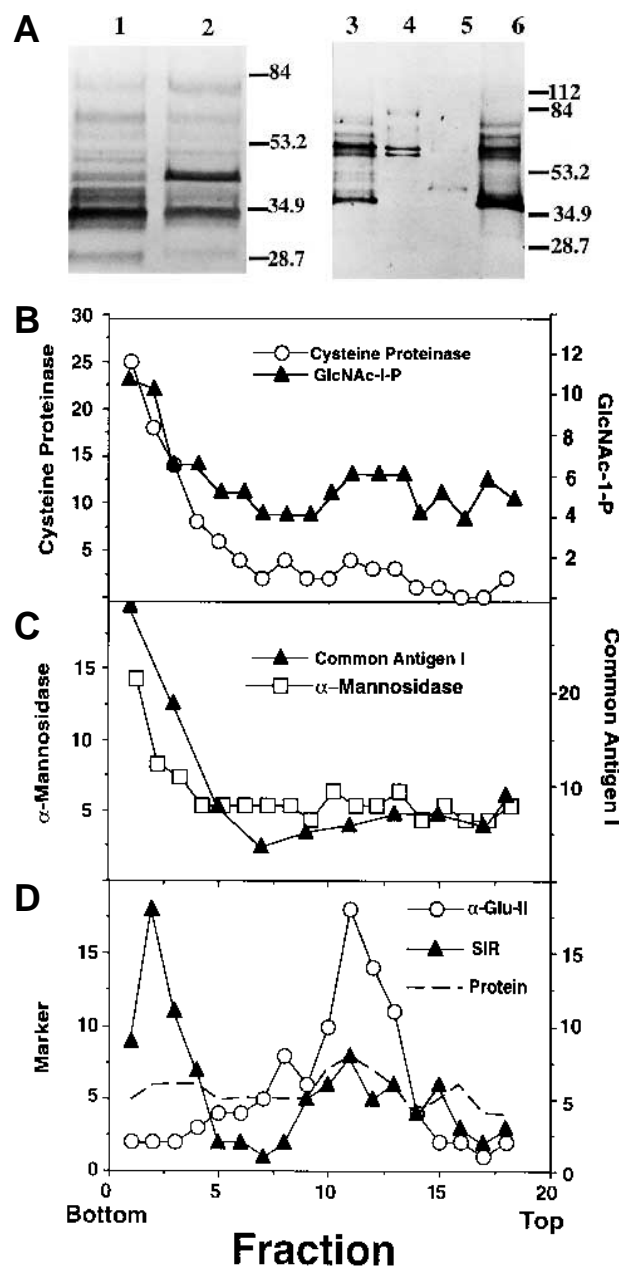


Fig. 1. GlcNAc-1-P-containing proteins and cysteine proteinases cofractionate with other lysosomal hydrolases. The PNS from AX2 cells was fractionated on a 24% Percoll density gradient. Fractionation, marker enzyme assays, quantitation of GlcNAc-1-P and Man-6-S on proteins, SDS-PAGE and western-blot analysis were performed as detailed in Materials and Methods. (A) Western blot analysis using GlcNAc-1-P antibody AD7.5 (lane 1, 7 μg of fraction 1; lane 2, 7 μg of fraction 11) and Man-6-S antibody mLE2 (lanes 3-6). Lane 3, 2 μg of protein from fraction 1; lane 4, 0.2 μg of partially purified α -mannosidase; lane 5, 1 μg of purified proteinase-1; lane 6, 10 μg of protein from PNS. (B) Profile of cysteine proteinase activity and GlcNAc-1-P content determined by ELISA. (C) Profile of α -mannosidase activity and Man-6-S content by ELISA. (D) Distribution of marker enzymes for mitochondria (SIR, succinate-INT-reductase), endoplasmic reticulum (α -Glu-II, α -glucosidase-II) and protein. A Golgi marker (Ser:GlcNAc phosphotransferase) fractionated identically to α -Glu-II (not shown). All enzyme activities and antigen reactivity are expressed as percentage of total. Bars indicate molecular mass (kDa) of marker proteins.

1984). mLE2 reactivity showed a typical lysosomal distribution (Fig. 1C), in agreement with previous studies (Judelson and Dimond, 1988). ELISA for Man-6-P-OCH₃ in Percoll gradient fractions could not be done due to technical difficulties. mLE2 recognizes similar bands in all gradient fractions (Fig. 1A, lane 3), and α -mannosidase (Fig. 1A, lane 4) is clearly one of the major proteins in the lysosomal and PNS fractions (Fig. 1A, lanes 3 and 6). Although proteinase-1 is weakly recognized by mLE2 (Fig. 1A, lane 5), it is not present in axenic cells. We conclude that the GlcNAc-1-P modification and CPs cofractionate with lysosomes and endosomes.

GlcNAc-1-P-modified proteins reside in functional vesicles of the endo-lysosomal pathway

Magnetic fractionation of vesicles containing iron-coated dextran has been used to purify and characterize functional *Dictyostelium* lysosomes and endosomes (Rodriguez-Paris et al., 1993; Temesvari et al., 1994). The results are comparable to those using FITC-dextran as a marker. Since Percoll fractionation indicated that phosphoglycosylated proteins are endo-lysosomal, we wanted to determine if the GlcNAc-1-P-containing proteins and the CPs reside in vesicles that can be functionally recruited to this pathway. Cells were incubated with iron-dextran for 2 hours to label the vesicles of the entire pathway at equilibrium (Rodriguez-Paris et al., 1993). After correcting for enzymes solubilized during homogenization, the iron-containing vesicles accounted for 70-85% of the various Man-6-P-OCH₃-containing lysosomal hydrolases and 84% of CP activity compared to the total activity of the PNS. The endo-lysosome fraction was 6- and 10-fold enriched in lysosomal glycosidase and CP activities, respectively, compared to the PNS. Densitometric scans of immunoblots probed with AD7.5 and mLE2 also showed 6.5- and 10-fold enrichment of glycoproteins containing these modifications, respectively (Fig. 2). Since most of the CP and glycosidase activities were recovered after magnetic fractionation, we conclude that these enzymes are in vesicles that are recruited to the endo-lysosomal pathway and that paramagnetic fractionation can be used to purify proteins modified by GlcNAc-1-P and Man-6-S.

Most of the axenic CPs are phosphoglycosylated

Because our past results showed that AD7.5 precipitates all of the CP activity from bacterially grown cells (Mehta et al., 1996) and that CP4, CP5 and CP7 (proteinase-1) all contain GlcNAc-1-P when overexpressed in axenic cells (Souza et al., 1996; Ord et al., 1997), we expected that AD7.5 would also precipitate all of the CP activity from axenic cells. However, the antibody precipitated only approx. 20-40% of the proteolytic activity from axenic cells that was sensitive to the CP-specific inhibitor, E-64 (Fig. 3A). Incomplete precipitation of the CP activity was not due to the presence of inhibitors in the extract since the antibody completely precipitated purified phosphoglycosylated proteinase-1 when added to the cell extract. Blots of the solubilized immunoprecipitate with AD7.5 showed that most of the reactive bands are precipitable, except for a prominent band at 29 kDa (Fig. 3A, inset).

To determine if CP precipitation was selective, active CPs from axenic lysosome extracts were applied to SDS gels without heating to preserve proteolytic activity. Following electrophoresis, the gel was incubated with a fluorogenic

peptide substrate and six bands of activity were seen at 43, 40, 37, 32, 29 and 20 kDa (Fig. 3B, lane 1). The bands were divided into five regions (I-V) and each was eluted with >90% recovery, completely denatured by boiling in SDS and analyzed by SDS/PAGE for GlcNAc-1-P by probing the immunoblots with AD7.5. All of these active CPs contained GlcNAc-1-P, except for band V (Fig. 3B). Note that complete denaturation causes some of the bands (I and II) to change their apparent molecular mass. This is probably due to a conformation difference or the Ser-rich domains found on some CPs. Similar changes are also seen when purified proteinase-1 is analyzed under these conditions. Activity bands of proteinase-1 run at 43, 37 and 33 kDa, but all run at 33 kDa when completely denatured (not shown).

Band V (20 kDa) was not precipitated by AD7.5, but it accounts for only 15% of the total CP activity. On the other hand, the failure to precipitate the GlcNAc-1-P-containing 29 kDa band (IV) with AD7.5 is more significant, since it accounts for approx. 50% of the total CP activity (Fig. 3B). Together the 20 and 29 kDa bands may account for the limited precipitation of CP activity. Although AD7.5 was unable to precipitate all of the axenic CP activity, most of the CPs contain GlcNAc-1-P.

Man-6-P-OCH₃/Man-6-S and GlcNAc-1-P are mutually exclusive post-translational modifications of *Dictyostelium* lysosomal proteins

To determine whether GlcNAc-1-P and Man-6-S are mutually exclusive modifications, axenic cell extracts were immunoprecipitated with either mAb AD7.5 or mLE2 and the precipitated proteins were probed for each modification. Fig. 4 shows that the GlcNAc-1-P-specific antibody, AD7.5, immunoprecipitates proteins (left panel, lane a) that are different from those

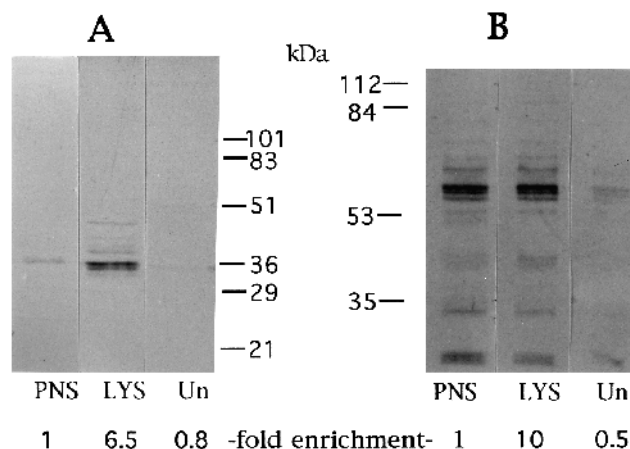


Fig. 2. GlcNAc-1-P-containing proteins and cysteine proteinases are recruited to the endo-lysosomal pathway. The entire endo-lysosomal pathway in axenic cells was labeled with iron dextran for 2 hours and the vesicles purified by magnetic fractionation. (A) 2 μ g each of postnuclear supernatant (PNS), lysosomes (LYS) and unbound fraction (Un) were analyzed by western blot using AD7.5 for GlcNAc-1-P. (B) 2 μ g of lysosomes and 20 μ g each of PNS and unbound fractions were probed with mLE2 for Man-6-S. 10 times more protein from PNS and unbound fractions was necessary to see the staining with mLE2. The numbers below the blots denote the fold-purification over PNS obtained after densitometric scans of the blots.

immunoprecipitated by the Man-6-S specific antibody, mLE2 (right panel, lane m). None of the same proteins was precipitated by both the antibodies. This is consistent with our previous observations using bacterially grown cells (Mehta et al., 1996). Since these blots were detected with anti-mouse conjugates, background IgG bands from the immunoprecipitation are also stained (Fig. 4, middle panel, lanes m and a). None of these bands was derived from the cell lysate (not shown).

We next wanted to determine whether any of the lysosomal proteins contained both GlcNAc-1-P and Man-6-S/Man-6-P-OCH₃. Vesicles purified from cells pulsed with iron-dextran for 15 minutes and chased for 15 minutes are dense, acidic, and

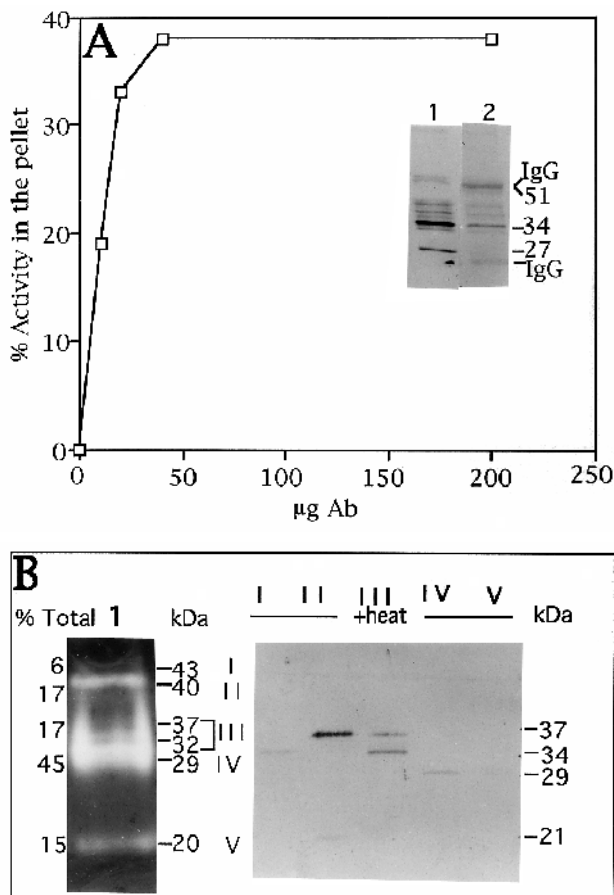


Fig. 3. The majority of the cysteine proteinases from *Dictyostelium* have GlcNAc-1-P. (A) Immunoprecipitation of CP activity from 20 µg of axenic cell extracts with increasing amounts of AD7.5. Inset: 5 µg of axenic cell extract was precipitated with AD7.5 and the pellet was analyzed by western blot and probed with AD7.5. Lane 1, 5 µg of axenic cell extract (control) and lane 2, proteins precipitated with AD7.5. The apparent molecular mass (kDa) of the marker proteins and light and heavy chains of the immunoglobulin are marked. (B) Lysosomal extract from axenic cells was separated by SDS/PAGE under non-heat-denaturing conditions and analyzed for CP activity with a fluorescent peptide substrate. The individual CPs were eluted from the gel as described in Materials and Methods. Each eluted band was analyzed by SDS/PAGE (+heat) and probed for GlcNAc-1-P with AD7.5. Lane 1 depicts the activity profile and % total CP activity is shown on the left side. The apparent molecular mass of bands in kDa is shown on the right side for lane 1 and the Roman numerals in lane 1 indicate the number assigned to the activity band that was eluted from the gel and analyzed with AD7.5.

enriched in Man-6-P-OCH₃-containing acid hydrolases and V-H⁺-ATPase activity, and therefore have the properties of mature lysosomes (Rodriguez-Paris et al., 1993; Temesvari et al., 1994). Lysosomes were prepared using this method and probed for GlcNAc-1-P, Man-6-S or Man-6-P-OCH₃. Fig. 5 shows that phosphoglycosylated proteins in whole lysosomes do not overlap with proteins containing phosphorylated (Man-6-P-OCH₃) or sulfated (Man-6-S) oligosaccharides. Binding of both monoclonal antibodies and the CI-MPR was specific, as shown by competition with 10 mM UDP-GlcNAc, Man-6-S or Man-6-P. Non-coincident patterns are also seen using soluble lysosomal contents or stripped lysosomal membranes. The blot of lysosomal contents (Fig. 5B) was overdeveloped to show the similarity in staining for Man-6-P-OCH₃- and Man-6-S-containing proteins, although some differences in intensities were observed. The apparent overlap of a 36 kDa band in lysosomal contents (Fig. 5B) results from overdevelopment of the blot since it is not seen when the blot is developed in the linear range (Fig. 5A). Thus, both specific immunoprecipitation from axenic cell lysates and immunoblots of purified lysosomes, their contents, or washed lysosomal membranes both show that GlcNAc-1-P and Man-6-S/Man-6-P-OCH₃ are mutually exclusive modifications.

GlcNAc-1-P and Man-6-P-OCH₃ define different sets of vesicles

Cells were analyzed by confocal immunofluorescence microscopy to see if the mutually exclusive modifications reflect differential intracellular distributions. Axenically grown cells were fixed and incubated with either AD7.5 and FITC-labeled anti-mouse IgG or with CI-MPR and TRITC-labeled anti-rabbit IgG. Fig. 6a, shows that GlcNAc-1-P-containing vesicles (green) do not colocalize with Man-6-P-OCH₃-containing ones (red). No yellow (red + green) vesicles, representing a possible colocalization of both sugars, was ever detected in >100 cells examined. The rare yellow vesicles seen in Fig. 6a are the result of overlaying all sections together since they are not observed when viewing individual sections or by tri-dimensional rotation of the cell images. Competition of AD7.5 with UDP-GlcNAc (Fig. 6c) shows that the green fluorescence (GlcNAc-1-P) disappears, but the red fluorescence

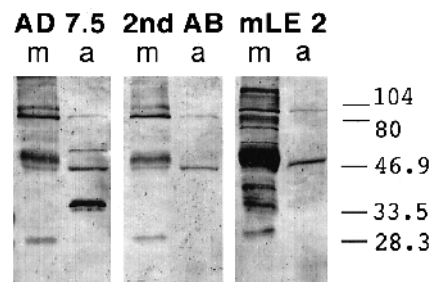


Fig. 4. Immunoprecipitation of different proteins by mAbs AD7.5 and mLE2. Axenic cell extract was immunoprecipitated with either AD7.5 for GlcNAc-1-P (a) or mLE2 for Man-6-S (m) as detailed in Materials and Methods. The precipitated proteins were separated by SDS-PAGE and analyzed on western blots with either AD7.5 or mLE2. Control for background staining from IgG bands with the secondary antibody is shown as 2nd AB. None of these bands was present in the cell extract. All were due to the antibody. Bars indicate molecular mass of marker proteins (kDa).

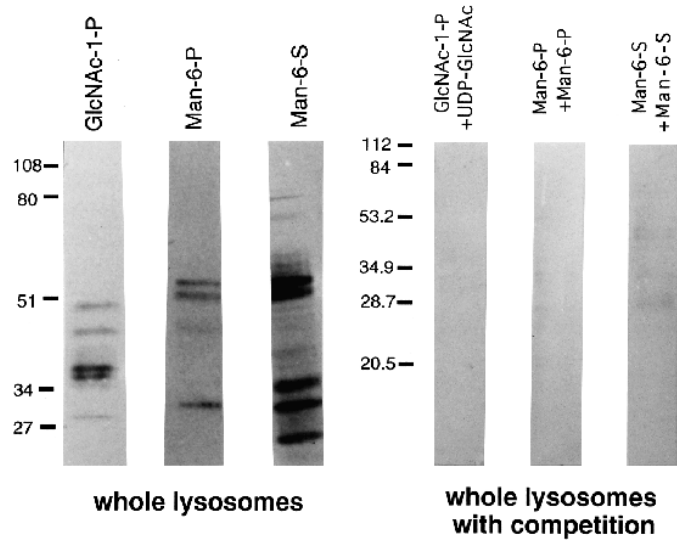


Fig. 5. GlcNAc-1-P and Man-6-P-OCH₃ define different sets of lysosomal enzymes. Axenic cells were fed colloidal iron-dextran for 15 minutes and chased in the absence of the probe for 15 minutes, and lysosomes were subsequently isolated by magnetic fractionation as detailed in Materials and Methods.

Whole lysosomes, lysosomal contents and stripped lysosomal membranes were analyzed by western-blot for GlcNAc-1-P (AD7.5), Man-6-P-OCH₃ (CI-MPR and anti-CI-MPR) and Man-6-S (mLE2). Specificity of the antibody binding in whole lysosomes was shown by adding 10 mM of UDP-GlcNAc, Man-6-P and Man-6-S to the respective primary antibody solutions. Lysosomal contents were analyzed with low protein (0.5 µg) to show that GlcNAc-1-P- and Man-6-P-OCH₃-containing proteins do not overlap (A) and also at high protein (5 µg) to show coincidence of Man-6-P-OCH₃ and Man-6-S bands (B). Protein load was 0.5 µg for whole lysosomes and 10 µg for stripped lysosomal membranes for all the antibodies. The apparent molecular mass (kDa) of the marker proteins is indicated.

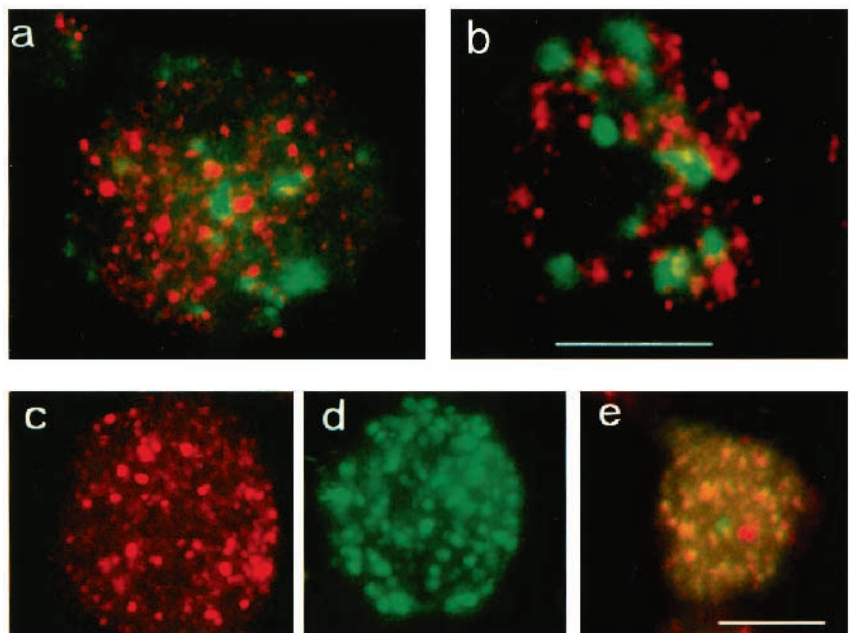
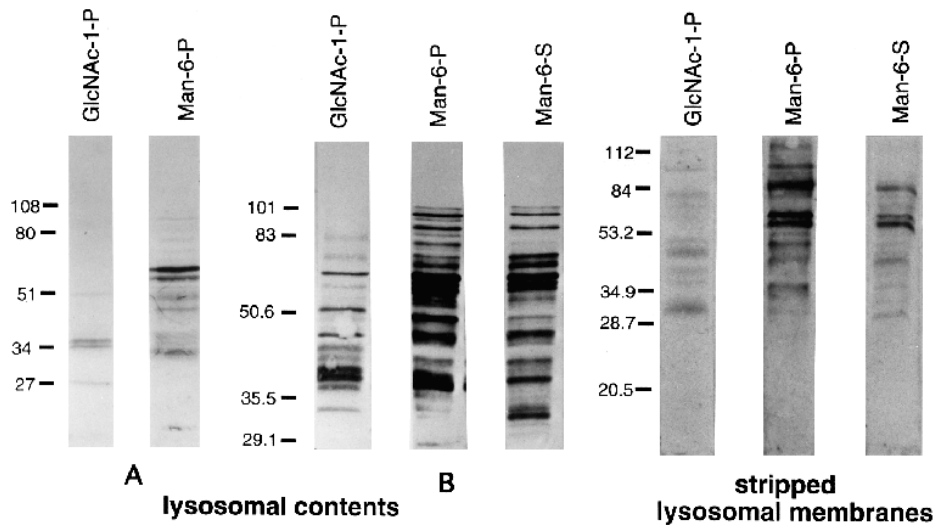


Fig. 6. Glycoproteins with GlcNAc-1-P and Man-6-P-OCH₃ are segregated into different vesicles in *Dictyostelium*. Exponentially grown AX-2 cells were washed, plated on coverslips and subjected to confocal immunofluorescence analysis as described under Material and Methods. GlcNAc-1-P-containing proteins were detected with mAb AD7.5, Man-6-P-OCH₃-containing proteins with CI-MPR/anti-CI-MPR and α -mannosidase with mAb 6H10. mAb AD7.5 and mAb 6H10 binding was detected with anti-mouse FITC-conjugate and anti-CI-MPR was detected with anti-rabbit-TRITC-conjugate. (a) Double staining of axenic cells with AD7.5 (green) and CI-MPR/anti-CI-MPR (red). (b) Vegetative cells stained as in a. (c) Same as in a, in the presence of 10 mM UDP-GlcNAc. (d) Same as in a, in the presence of 10 mM Man-6-P. (e) Double staining with CI-MPR/anti-CI-MPR (red) and 6H10 (green). Bar, 5 µm.

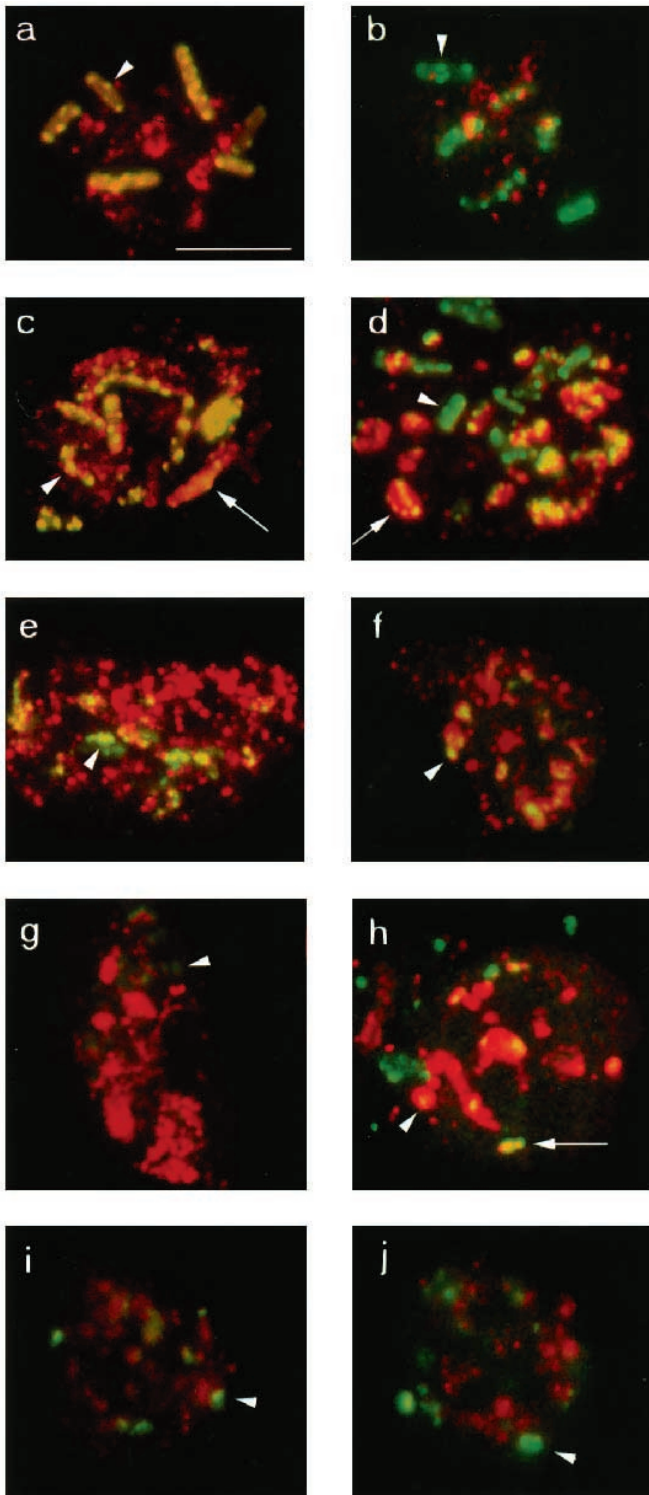


Fig. 7. Sequential fusion of phagosomes with GlcNAc-1-P- and Man-6-P-OCH₃-containing vesicles. Axenic cells were harvested, washed and pulsed with FITC-bacteria for 3 minutes (a and b) or 15 minutes (c and d). Chases in buffer were performed for different times: (a-d) no chase; (e and f) 15 minutes chase; (g and h) 30 minutes chase; (i and j) 60 minutes chase. At different time points cells were plated for confocal immunofluorescence microscopy, stained with AD7.5 (left panels) and CI-MPR/anti-CI-MPR (right panels), and analyzed with anti-mouse-IgG-TRITC and anti-rabbit-IgG-TRITC conjugates, respectively. Bar, 5 μ m.

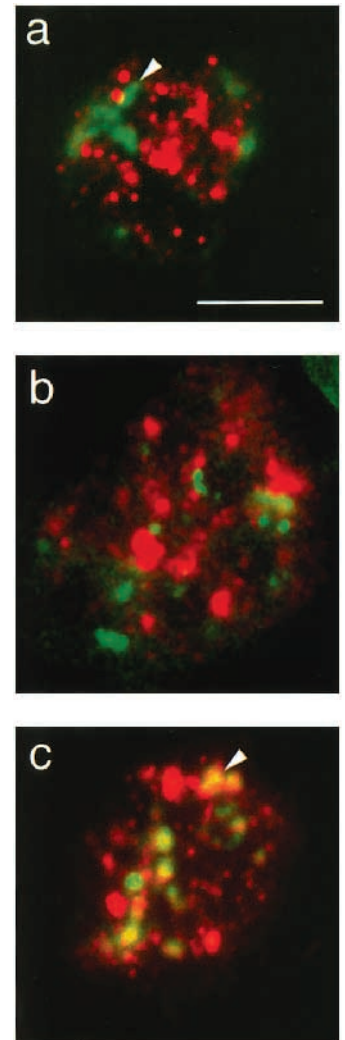


Fig. 8. Glycoproteins with GlcNAc-1-P and Man-6-P-OCH₃ remain mostly segregated during bacterial digestion. Cells were pulsed with non-fluorescent bacteria for 3 minutes (a) and chased for 30 minutes (b and c). Cells were double-stained with AD7.5 (detected with anti-mouse FITC-conjugated IgG) and with the CI-MPR/anti-CI-MPR (detected with anti-rabbit TRITC-conjugated IgG). (b) In 95% of the cells GlcNAc-1-P and Man-6-P-OCH₃ is not colocalized; (c) some colocalization of the two modifications occurs in approx. 5% of the cells. Bar, 5 μ m.

(Man-6-P-OCH₃) remains. Conversely, Fig. 6d shows that incubating CI-MPR with Man-6-P eliminates the red fluorescence, but not the green (GlcNAc-1-P). To show that a known lysosomal enzyme marks the Man-6-P-OCH₃-containing vesicles, we used a peptide-specific antibody (mAb 6H10) against lysosomal α -mannosidase (Mierendorf and Dimond, 1983) and FITC-labeled second antibody (green). As shown for Fig. 6e, >80% of the Man-6-P-OCH₃ vesicles contain this enzyme and are yellow. These observations indicate that proteins containing GlcNAc-1-P and Man-6-P-OCH₃ reside in different lysosomal compartments. Cells grown in axenic medium contain about 200 vesicles of each type that have diameters ranging from 200-500 nm. Vesicles are randomly scattered throughout the cell, while nuclei and cell surfaces do not stain.

A similar segregated pattern of GlcNAc-1-P and Man-6-P-OCH₃ vesicles is also seen on cells grown on bacteria, as shown in Fig. 6b.

GlcNAc-1-P- and Man-6-P-OCH₃-containing compartments sequentially attack bacterial phagosomes

Since *Dictyostelium* is very efficient in phagocytosing bacteria

(Glynn, 1981) we wanted to investigate whether the GlcNAc-1-P- and/or Man-6-P-OCH₃-containing lysosomal vesicles fuse with phagosomes to carry out digestion. To test this possibility, cells in axenic medium were fed FITC-labeled bacteria for 3 minutes or 15 minutes and then, in the latter case, chased in the absence of bacteria for up to 1 hour. Representative results from observing at least 50 cells stained with each antibody at each time point are shown in Fig. 7. In the left column (a,c,e,g,i) cells were incubated with the GlcNAc-1-P antibody and detected with an anti-mouse TRITC-IgG. In the right column (b,d,f,h,j) cells were incubated with the CI-MPR and anti-CI-MPR followed by an anti-rabbit TRITC-IgG. After a 3-minute pulse, all of the FITC-bacteria in phagosomes are yellow, showing that they fuse with the GlcNAc-1-P-containing vesicles (a, arrowhead). In contrast, there is no colocalization of bacteria with Man-6-P-OCH₃-containing vesicles at 3 minutes since all of the bacteria remain green (b, arrowhead).

The exclusive colocalization of AD7.5 reactivity and bacteria is not due to binding of the antibody to the bacteria themselves, since incubation of the antibodies with bacteria alone did not produce any staining (not shown). After a 15-minute pulse with bacteria, and in all of the subsequent chase points, there is a gradual shift of green fluorescent bacteria from the GlcNAc-1-P compartments into the Man-6-P-OCH₃-containing vesicles. The timing of this event seems to be heterogeneous; the cells shown in the remaining panels of Fig. 7 are representative of 70% of the population.

After a 15-minute pulse, the FITC-bacteria appear to be partially digested since they lose their sharp, distinct borders (c, arrowhead) and in some instances vesicles stain less intensely (c, arrow). By this time (15-minute pulse), some of the bacteria appear to have fused with the Man-6-P-OCH₃ compartment to form a yellow vesicle (d, arrow); however, a substantial portion of the bacteria are not colocalized with Man-6-P (d, arrowhead). After a 15-minute chase, some green fluorescence starts to be seen in cells stained for GlcNAc-1-P (e, arrowhead), but the majority of vesicles still remain yellow. Cells stained for Man-6-P-OCH₃ show little green fluorescence, indicating that the bacteria are now colocalized with the Man-6-P-OCH₃-containing material (f, arrowhead). Chasing for 30 minutes produces an increase in green fluorescence in the cells stained for GlcNAc-1-P (g, arrowhead) as well as Man-6-P-OCH₃ (h, arrow). However, bacteria or fragments are still found in some Man-6-P-OCH₃ vesicles (h, arrowhead). Some cells seem to take longer to digest all the bacteria and occasionally (10%) intact green bacteria (d, arrowhead) are seen at 15 and 30 minutes of chase (not shown). These may be bacteria that were phagocytosed at the end of the pulse and have not left the GlcNAc-1-P compartments. After 1 hour of chase, almost none of the residual FITC label colocalizes with vesicles containing either GlcNAc-1-P or Man-6-P-OCH₃ (i and j, arrowheads).

Axenic cells were also pulsed for 3 minutes with FITC-dextran, FITC-latex beads or magnetic beads. However, none of these probes had a temporal preference for fusion with GlcNAc-1-P-containing vesicles (data not shown). At 3 minutes, approx. 10% of vesicles containing FITC-dextran or FITC-latex beads colocalized with TRITC-labels for both GlcNAc-1-P and Man-6-P-OCH₃. Immunoblots of vesicles purified after a 3-minute pulse with magnetic beads also showed no preferential enrichment of GlcNAc-1-P- over Man-

6-P-OCH₃-containing proteins. Thus, the amoebae are able to specifically distinguish bacteria from artificial probes.

Colocalization of GlcNAc-1-P and Man-6-P-OCH₃ is rarely seen during phagocytosis of bacteria

Regardless of the food source, GlcNAc-1-P- and Man-6-P-OCH₃-containing proteins are hardly ever colocalized. Since bacteria seem to be associated with GlcNAc-1-P-containing compartments first, and later with Man-6-P-OCH₃-containing compartments, one would expect these two modifications to be colocalized at some point. We have not been able to detect such a colocalization in cells continuously fed bacteria, as shown in Fig. 6b. Since we are unable to do three-color colocalization to follow the digestion of FITC-labeled bacteria and both sugar-modifications in the vesicles, we synchronized a population of cells with a pulse of non-fluorescent bacteria for 3 minutes, and then chased to search for vesicles that contained both GlcNAc-1-P (green) and Man-6-P-OCH₃ (red). Over 50 cells were analyzed at each time point. Fig. 8a shows a cell that was incubated with bacteria for 3 minutes. The GlcNAc-1-P vesicles adopt an elongated form (arrowhead) and appear to be fused with the phagosomes, similar to that seen in Fig. 7a. After a 30-minute chase, 95% of cells do not show any overlap of GlcNAc-1-P and Man-6-P-OCH₃ (Fig. 8b); however, overlap is seen in a few vesicles in approx. 5% of the cells (Fig. 8c, arrowhead). This probably means that the apparent colocalization is either a very rare event or a short-lived association, although we cannot eliminate the possibility that colocalization does occur and is below our detection limits.

Lysosomal enzymes and their carbohydrate modifications are stable

Another explanation for the lack of colocalization could be loss of both GlcNAc-1-P and Man-6-P-OCH₃, due to degradation of the sugar modifications or degradation of the proteins themselves. To examine this, purified phosphoglycosylated proteinase-1 was incubated at either pH 4.5 or 6.0 for 24 hours. There was no loss in proteinase activity, change in mAb AD7.5 reactivity, or appearance of additional bands on SDS/PAGE that might signal auto-proteolysis (not shown). Moreover, incubation of a peptide containing [³H]GlcNAc-1-P-Ser with 5 mg/ml of lysed lysosome or endosome fractions at pH 5.0 or 7.0 for up to 48 hours showed <3% release of [³H]GlcNAc or GlcNAc-1-P.

DISCUSSION

Our results indicate the presence of two sets of lysosomal proteins with mutually exclusive carbohydrate modifications in *Dictyostelium* (Figs 4 and 5). GlcNAc-1-P- and Man-6-P-OCH₃-containing glycoproteins are sorted to two sub-populations of endo-lysosomal vesicles (Figs 6-8). The segregation of these two groups of glycoproteins may have physiological significance. The results suggest the following sequence of events: first, phagosomes loaded with bacteria fuse with a compartment containing GlcNAc-1-P-modified proteins, and after an initial digestion, presumably by CPs, they fuse with vesicles containing Man-6-P-OCH₃-modified proteins. Further digestion occurs and the bacterial fragments are then found in compartments that do not seem to contain either of the sugar

modifications. No colocalization of the two modifications occurs under our conditions.

Subcellular fractionation indicated that the majority of the GlcNAc-1-P-containing proteins and CP activity cofractionate with typical lysosomal enzymes (Fig. 1). Magnetic fractionation of the endo-lysosomal vesicles showed that vesicles containing these proteins are recruited to the endo-lysosomal pathway (Fig. 2). Based on previous work (Mehta et al., 1995, 1996; Souza et al., 1995) we expected that all CPs from axenic cells would also have GlcNAc-1-P and be precipitated with AD7.5. While most contained GlcNAc-1-P, the antibody precipitated a low proportion of CP activity (Fig. 3A). This was primarily because the most active GlcNAc-1-P-containing CP (29 kDa) was not precipitable by AD7.5 (Fig. 3A, inset). This proteinase is very likely be CP5, based on its apparent molecular mass and the presence of GlcNAc-1-P (Souza et al., 1995). Some CPs such as the 20 kDa activity band (Fig. 3B) do not appear to contain GlcNAc-1-P. At least two additional partially sequenced cDNAs from axenic cells that code for CPs lack the typical serine-rich domain where GlcNAc-1-P could be added (Urushihara, H., server-<http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html>, accession number-FC-AH21 and FC-AJ13). Finally, some GlcNAc-1-P-reactive proteins seen in axenic cells may not be active CPs.

The early fusion with the phagocytosed bacteria (<3 minutes) (Fig. 7) suggests that GlcNAc-1-P-containing proteins are present in endosomes. This is consistent with the peak of AD7.5 reactivity in the endosomal region of the Percoll fractions and the presence of 16% of the total CP activity (Fig. 1b). *Dictyostelium* cysteine proteinases have considerable activity at the endosomal pH of 6.5 and recently a 34 kDa cysteine proteinase was isolated from *Dictyostelium* endosomes (Adessi et al., 1995). Endosomes from mammalian cells also contain cysteine and aspartyl proteases, suggesting that some digestion may already occur in this mildly acidic compartment (Runquist and Havel, 1991; Casciola-Rosen et al., 1992). Azurophil granules from neutrophils also contain proteases to degrade bacteria in these specialized compartments (Bainton, 1992).

Phagosomes prepared from cells pulsed for 3 minutes with 1 μ m magnetic beads showed no enrichment of phosphoglycosylated proteins over those containing Man-6-P-OCH₃. Furthermore, confocal microscopy of cells fed with FITC-latex beads or FITC-dextran for 3 minutes showed colocalization with both GlcNAc-1-P- and Man-6-P-OCH₃-containing vesicles, suggesting that distinct segregation may occur only during the phagocytosis of bacteria. In professional phagocytes like macrophages, the nature of the phagocytosed particle affects the early fusion events with lysosomes, the dynamics of the phagolysosome formation and also the intracellular half life of particle-associated proteins (Oh and Swanson, 1996).

Essentially, all of the Man-6-P on *Dictyostelium* enzymes occurs as a stable phosphomethyldiester (Freeze et al., 1983; Freeze and Wolgast, 1986) and only a phosphodiesterase from *Aspergillus niger* is capable of degrading it (Gabel et al., 1984). *Dictyostelium* does not appear to have a similar activity (Freeze et al., unpublished results). Methyl capping of Man-6-P might be used to ensure its long-term retention after initial delivery to the lysosome, and then later for retrieval from phagosomes or other vesicles. The stability of the GlcNAc-1-P-Ser could also be important for a similar reason. It is possible, however,

that special conditions occur within phagosomes that degrade these carbohydrates, or that the bacterial fragments are carried into a compartment lacking either modification.

How could the segregation/sorting of these two carbohydrate modifications be achieved? The cell biology and oligosaccharide structures of Man-6-P-OCH₃-containing lysosomal enzymes have been extensively studied in normal and mutant cell lines in *Dictyostelium* (Cardelli, 1993; Freeze, 1990), but the question of lysosomal enzyme targeting and retrieval is unresolved. Proteolytic processing of newly synthesized α -mannosidase and β -glucosidase is clearly critical, since cells incubated with cysteine/serine protease inhibitors secrete unprocessed precursors, rather than delivering them to dense lysosomal vesicles (Cardelli et al., 1986; Richardson et al., 1988). Even though the mammalian CI-MPR recognizes *Dictyostelium* lysosomal enzymes (Freeze et al., 1980), no such receptors have been identified in *Dictyostelium* and the role of Man-6-P-OCH₃ in lysosomal enzyme targeting/retrieval is still questionable. A Man-6-P-independent mechanism must therefore exist to target the phosphoglycosylated proteins to the lysosomes such as those elucidated in mammalian cells (McIntyre and Erickson, 1993; Kornfeld and Mellman, 1989; Marcusson et al., 1994).

Maintenance of the segregation may require selective retrieval of the proteins during sequential fusions of different vesicles with the phagosome, as suggested in higher organisms (Rabinowitz et al., 1992; Beron et al., 1995; Desjardins, 1995). The enrichment of cathepsins B and D in hepatocyte membranes and in macrophage endosomes (Diment et al., 1988; Authier et al., 1995) is proposed to be due to the recovery of these enzymes through membrane recycling to endosomes via undefined receptors (Kornfeld, 1986; von Figura and Hasilik, 1986). Likewise, a retrieval system for the cysteine proteinases, using a receptor that would perhaps recognize GlcNAc-1-P, is conceivable. Distinct vacuolar distribution of a CP, aleurain, and a lectin in barley root tip cells suggests that distinct vesicular segregation of lysosomal proteins may also be relevant to other systems (Paris et al., 1996). Further work on the mechanism used to establish and maintain the two different compartments in *Dictyostelium* should provide insights into many aspects of sorting, phagocytosis and lysosome biogenesis.

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