Specific alterations in levels of mannose 6-phosphorylated glycoproteins in different neuronal ceroid lipofuscinoses

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Mannose 6-phosphate (Man-6-P) is a carbohydrate modification that is generated on newly synthesized lysosomal proteins. This modification is specifically recognized by two Man-6-P receptors that direct the vesicular transport of the lysosomal enzymes from the Golgi to a prelysosomal compartment. The Man-6-P is rapidly removed in the lysosome of most cell types; however, in neurons the Man-6-P modification persists. In this study we have examined the spectrum of Man-6-P-containing glycoproteins in brain specimens from patients with different neuronal ceroid lipofuscinoses (NCLs), which are progressive neurodegenerative disorders with established links to defects in lysosomal catabolism. We find characteristic alterations in the Man-6-P glycoproteins in specimens from late-infantile (LINCL), juvenile (JNCL) and adult (ANCL) patients. Man-6-P glycoproteins in LINCL patients were similar to controls, with the exception that the band corresponding to CLN2, a recently identified lysosomal enzyme whose deficiency results in this disease, was absent. In an ANCL patient, two Man-6-P glycoproteins were elevated in comparison with normal controls, suggesting that this disease also results from a perturbation in lysosomal hydrolysis. In JNCL, total levels of Man-6-P glycoproteins were 7-fold those of controls. In general this was reflected by increased lysosomal enzyme activities in JNCL but three Man-6-P glycoproteins were elevated to an even greater degree. These are CLN2 and the unidentified proteins that are also highly elevated in the ANCL.

INTRODUCTION

The neuronal ceroid lipofuscinoses (NCLs) are a group of progressive hereditary neurodegenerative diseases that affect infants, children and adults [1]. The pathological hallmark of these disorders is the accumulation of an autofluorescent pigment (ceroid-lipofuscin) in lysosome-like bodies within the neurons and other cells of patients. The ultrastructural appearance of the stored material, when considered with age of onset and clinical course, defines three major childhood forms of NCL and one adult form.

The molecular bases for several forms of NCL have been recently discovered. By linkage analysis and screening of positional candidates, the defective gene (*CLN1*) in infantile NCL (Santavuori–Haltia disease) has been identified as palmitoyl-protein thioesterase (PPT1) [2], an enzyme involved in the removal of the lipid moiety from *S*-acylated proteins [3]. By using a biochemical approach, the defective gene (*CLN2*) in classical late-infantile NCL (LINCL; Jansky–Bielschowsky disease) was identified as an unusual and previously unknown pepstatin-insensitive protease [4]. Finally, the defective gene (*CLN3*) in juvenile NCL (JNCL; Batten disease; Vogt–Spielmeyer disease), which encodes a 438-residue protein of currently unknown function, was identified by linkage analysis [5].

Each of the three identified proteins whose deficiency results in NCL are localized within the lysosome [4,6–9]. Whereas the CLN3 protein seems to be a transmembrane lysosomal protein [5,10], the CLN2 protease and palmitoyl-protein thioesterase are soluble lysosomal enzymes. Newly synthesized soluble lysosomal enzymes contain a modified carbohydrate residue, mannose 6-phosphate (Man-6-P), which is recognized by two high-affinity Man-6-P receptors (MPRs), the cation-dependent MPR and the cation-independent MPR (CI-MPR). These MPRs direct the vesicular transport of the newly synthesized lysosomal enzymes from the Golgi to an acidified prelysosomal compartment where the receptor–ligand complex dissociates. In most cell types the Man-6-P moiety is rapidly removed [11] but in neurons lysosomal enzymes seem to retain the marker.

In this study, we have analysed Man-6-P glycoproteins in several NCLs. This has allowed us not only to determine whether specific alterations in lysosomal enzymes are associated with each type of NCL but also to address experimentally earlier suggestions [12] that there might be abnormalities in the removal of Man-6-P in NCLs. We find that each NCL shows characteristic alterations in the levels of Man-6-P glycoproteins. In particular, levels of Man-6-phosphorylated lysosomal enzymes are highly elevated in JNCL compared with normal. For most Man-6-P glycoproteins, increases in JNCL probably reflect increased lysosomal enzyme levels. For others, including the CLN2 protease, the magnitude of the increase is too great to be due simply to increased levels of active enzyme, suggesting alterations in the processing or transport of select lysosomal enzymes in JNCL.

EXPERIMENTAL

Materials

Control brains were autopsy samples from accident victims and were obtained from the National Disease Research Interchange (Philadelphia, PA, U.S.A.) (NDRI: no. 3497, 23-year-old male; no. 24625, 17-year-old female; no. 24490, 22-year-old male). Some of the JNCL samples were obtained from the National Neurological Research Specimen Bank (Los Angeles, CA, U.S.A.) (HSB 2244, 19-year-old male; HSB 2549, 34-year-old male; HSB 2431, 20-year-old female; HSB 2486, 30-year-old female). Two additional JNCL, four LINCL and the adult NCL

Abbreviations used: ANCL, adult NCL; CI-MPR, cation-independent MPR; JNCL, juvenile NCL; LINCL, late-infantile NCL; Man-6-P, mannose 6-phosphate; MPR, Man-6-P receptor; NCL, neuronal ceroid lipofuscinosis; sCI-MPR, soluble CI-MPR.

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(ANCL) brain samples were from the New York State Institute for Basic Research Collection (JNCL: H8754, 18-year-old female; B7089, 21-year-old male. LINCL: B11177, 6-year-old male; B140994, 7-year-old female; B8191, 10-year-old male. ANCL: B010777, 39-year-old male). Control fibroblasts GM-5757, GM408, GM323 and JNCL fibroblasts C-9282, C-6829 and C-4942 were from the Institute for Basic Research Collection.

SDS/PAGE and detection of Man-6-P glycoproteins

Extracts of autopsy samples were fractionated by electrophoresis on polyacrylamide gels and transferred to 0.2 μm pore-size nitrocellulose as described [13]. Man-6-P glycoproteins were detected with an iodinated soluble fragment of the CI-MPR [14].

Enzyme activity measurements

α-Galactosidase and α-galactosidase A activities were measured by using 5 mM 4-methylumbelliferyl-α-galactoside as a substrate in 0.1 M acetate buffer, pH 4.5, in the absence and the presence of 5 mM *N*-acetylgalactosamine respectively. Other lysosomal and control enzyme activities were measured as described previously [4,7,15].

Determination of proportion of lysosomal enzyme activities represented by the Man-6-phosphorylated form

The proportion of different enzymes which contained the Man-6-P targeting signal in different brain extracts was determined by activity measurements on the load, flow-through and Man-6-P eluate from analytical immobilized sCI-MPR affinity columns, essentially as described previously [7]. In brief, 150–190 mg of each sample was homogenized in 10 vol. of column buffer [CB; 25 mM Tris/HCl (pH 6.9)/150 mM NaCl/0.1 % (v/v) Triton X-100] and centrifuged at 40000 ***g*** for 30 min at 4 °C. A 1 ml sample of each supernatant (load) was applied to a column of immobilized sCI-MPR, which was then washed with 2 ml of CB and 3 ml of CB containing 5 mM glucose 6-phosphate. Bound Man-6-P glycoproteins were eluted with 6 ml of 10 mM Man-6-P. Enzyme activities were measured in the load (after dilution 1:6 in CB), the pooled flow-through and wash fractions and the Man-6-P eluate after each pool were adjusted to contain the same concentration of Man-6-P and glucose 6-phosphate. Specific activities were expressed relative to the protein concentration of the load supernatant [16]. The percentage of each activity corresponding to the Man-6-phosphorylated form was calculated as the Man-6-P eluate activity divided by the sum of the Man-6-P eluate activity and the flow-through plus wash activity. Average recovery (Man-6-P eluate plus flow-through plus wash divided by load) was 97 %. A 50 μl sample of each fraction was used for SDS/PAGE.

RESULTS

Mannose-6-phosphorylated glycoproteins in human NCLs

Detergent-soluble extracts were prepared from autopsy brain material from controls or different NCL patients, then fractionated by SDS/PAGE and transferred to nitrocellulose. Man-6-P glycoproteins were detected with an iodinated fragment of the CI-MPR as a probe in a Western blot-style assay. Results are presented in Figures 1 and 2. The signal was essentially abolished in the presence of 5 mM free Man-6-P, indicating that each band represented a Man-6-P-containing glycoprotein (Figure 1, left panel). For reference, seven prominent well-defined bands are indicated.

Distinct differences were observed in the levels of Man-6-P glycoproteins in each of the NCLs examined compared with those of normal controls. Man-6-P glycoproteins in LINCL were slightly elevated (overall levels 2.1-fold; individual bands 1.3–3.7-fold) relative to controls. In addition, a major band of approx. 46 kDa was absent from, or greatly diminished in, the LINCL extracts. This missing protein has been identified as the product of the CLN2 gene, which is defective in LINCL [4]. Another protein of slightly higher molecular mass than the CLN2 protein appears in the LINCL extracts that is not visible in the normal control. Whether this represents up-regulation of other lysosomal proteins or transient accumulation of a mutant CLN2 precursor remains to be determined.

In the five JNCL extracts, absolute levels of Man-6-P glycoproteins were significantly elevated (on average 8.7-fold) in comparison with controls (Table 1); however, not all Man-6-P glycoproteins were elevated to the same extent. In particular, bands 3, 5 and 6 were highly elevated (13.2-fold, 20.4-fold and 13.0-fold respectively). Band 3, the most prominent Man-6-P glycoprotein detected in human brain extracts, predominantly comprises the CLN2 protease. Levels of other bands, e.g. band 2, in JNCL were essentially the same as in the normal control [the small but significant apparent elevation of band 2 in JNCL [3.2 ± 1.0 (S.D.)-fold; Table 1] is due to a generalized increase in background in the region of this band]. Levels of Man-6-P glycoproteins in extracts from a patient with ANCL (Kuf's disease) were slightly elevated (2.5-fold) compared with those in the control, with the exception of two proteins of approx. 33 and 37 kDa (Figure 2, arrowed), which were highly elevated (11.9-fold and 15.0-fold) compared with controls.

Man-6-P glycoproteins in cell lines from NCL patients

To determine whether the elevated levels of Man-6-P glycoproteins observed in JNCL brain autopsy samples could be readily studied in a cultured cell system, we probed fractionated JNCL fibroblast extracts with iodinated CIMPR (Figure 2). One JNCL cell line exhibited slightly elevated (3.1-fold) levels of Man-6-P glycoproteins, but if the results are taken together the three JNCL cell lines did not contain significantly higher levels of Man-6-P glycoproteins than those in control cell lines.

Determination of phosphorylated proportion of lysosomal enzymes in NCLs

There are a number of cellular changes that could potentially result in increased levels of Man-6-P glycoproteins in JNCL. Synthesis of lysosomal enzymes might simply be increased in the disease state; this could be reflected by a concomitant increase in the Man-6-P-containing forms. Alternatively the newly synthesized lysosomal enzymes might not be dephosphorylated to the same extent in the disease state; thus the levels of lysosomal enzymes would remain the same but the fraction containing Man-6-P would be increased. This could result from a decreased activity of the currently unidentified Man-6-phosphatase or could reflect altered targeting to a Man-6-phosphatase-deficient compartment.

To determine whether either of these two possibilities was likely, the activities of 18 enzymes (16 lysosomal enzymes targeted by the Man-6-P pathway, one lysosomal enzyme targeted by a Man-6-P-independent pathway and one cytosolic enzyme) were determined in NCL and control brain extracts and the fraction of each activity represented by the Man-6-phosphorylated form was determined by MPR affinity chromatography. As a measure of the efficiency of purification of the Man-6-P glycoproteins on the immobilized MPR columns, samples of the loaded homogenate, the flow-through plus wash pool and the Man-6-P eluate

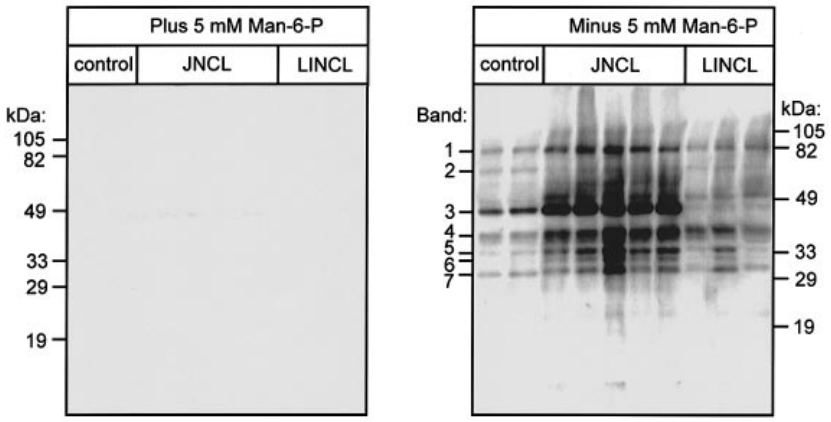

Figure 1 Man-6-P glycoproteins in extracts of brain samples from NCL patients

Total protein (50 μg) was fractionated on 10–20% (w/v) gradient polyacrylamide gels, transferred to nitrocellulose and Man-6-P glycoproteins were detected with an iodinated MPR affinity probe. In a control blot (left panel), the probing solution contained 5 mM Man-6-P in addition to the radiolabelled receptor to demonstrate the specificity of detection. Seven distinct bands are labelled 1–7 for the purposes of quantification (Table 1). The positions of molecular mass markers are indicated at the outer edges.

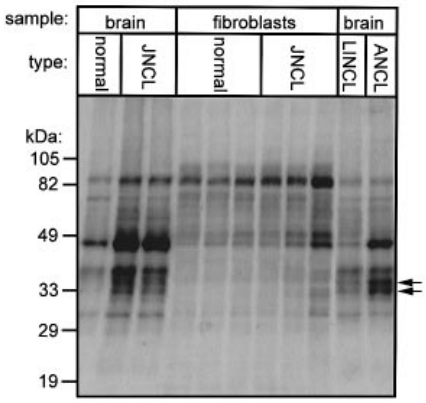

Figure 2 Man-6-P glycoproteins in extracts from brain samples and cell lines derived from NCL patients

Extracts were fractionated and probed with radiolabelled receptor as described in the legend to Figure 1. The two Man-6-P glycoproteins that are highly elevated in ANCL compared with control samples are indicated by arrows. The positions of molecular mass markers are indicated at the left.

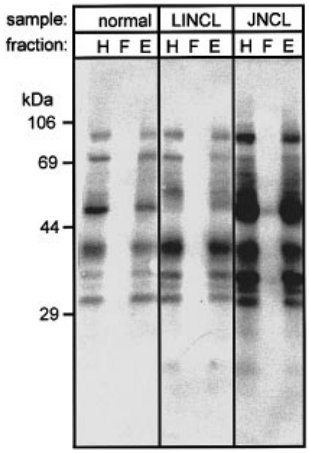

Figure 3 Man-6-P glycoproteins in NCL extracts subjected to immobilized MPR affinity chromatography

The preparation of extracts from two control brain samples, two LINCL brain samples and four JNCL brain samples and the analytical purification of Man-6-P glycoproteins by affinity chromatography on immobilized sCI-MPR were as described in the Experimental section. Equivalent volumes of each fraction were loaded on a 11.5% (w/v) polyacrylamide gel and Man-6-P glycoproteins were detected by using iodinated sCI-MPR. Fractions were homogenate (H), flow-through plus wash (F) and Man-6-P eluate (M). The positions of molecular mass markers are indicated at the left.

Table 1 Relative levels of individual Man-6-P glycoproteins in disease extracts relative to normal controls

Elevated levels of each band relative to controls were determined from three control samples, six JNCL samples and three LINCL samples. Results are means ± S.D.

	Glycoprotein level (% of control)	
Band	JNCL	LINCL
1	4.7 ± 1.4	1.7 ± 0.5
2	3.2 ± 1.0	1.3 ± 0.3
3	13.2 ± 2.4	0.3 ± 0.0
4	8.9 ± 3.7	3.7 ± 0.6
5	20.4 ± 22.1	3.3 ± 2.0
6	13.0 ± 13.0	2.8 ± 1.3
7	5.1 ± 4.0	1.9 ± 0.9
Total	8.7 ± 2.7	2.1 ± 0.4

were fractionated by SDS/PAGE and Man-6-P glycoproteins were detected with the radiolabelled MPR probe (Figure 3). For all extracts, essentially all of the Man-6-P glycoprotein content was recovered in the Man-6-P eluate.

On average, the activities of Man-6-P-containing lysosomal enzymes were elevated 2.2-fold in JNCL compared with control, which suggests that the elevated Man-6-P glycoprotein content could at least partly reflect elevated lysosomal enzyme synthesis (Table 2). This increase is certainly of a magnitude sufficient to account for elevated levels of some of the Man-6-P glycoproteins (e.g. bands 1, 2 and 7; Table 1) in JNCL but is insufficient to explain elevated levels of others. In LINCL, the 2.1-fold increase in levels of Man-6-P glycoproteins can simply be attributed to an

Table 2 Hydrolase activities and proportion represented by the Man-6-P-containing form in normal and NCL extracts

In parentheses in the last two columns, the total activity of each enzyme in JNCL and LINCL is expressed as a percentage of the normal value. Thimet oligopeptidase and acid phosphatase represent cytosolic and lysosomal enzymes respectively that do not contain Man-6-P. Abbreviation: n.d., not determined. Results are means ± S.D.

	Man-6-phosphorylated glycoprotein (% of total activity)			Total activity (nmol of substrate/h per mg of protein)		
Enzyme	Normal	LINCL	JNCL	Normal	LINCL	JNCL
β-Galactosidase	81 ± 3	81 ± 7	83 ± 2	50.9 ± 1.8	107.4 ± 27.9 (211)	75.2 ± 15.3 (148)
α-Glucosidase	34 ± 2	26 ± 3	22 ± 2	12.2 ± 0.4	38.2 ± 6.8 (313)	34.5 ± 12.4 (283)
α-Mannosidase	52 ± 5	57 ± 7	55 ± 5	2.1 ± 0.6	3.2 ± 0.5 (152)	5.1 ± 2.7 (243)
β-Mannosidase	65 ± 3	63 ± 9	71 ± 4	17.9 ± 12.9	28.1 ± 0.7 (157)	23.0 ± 0.5 (128)
β-Glucuronidase	47 ± 8	47 ± 2	49 ± 8	16.2 ± 22.6	73.6 ± 15.1 (454)	66.4 ± 39.3 (410)
Hexosaminidase	70 ± 5	76 ± 5	71 ± 3	18.5 ± 5.4	33.9 ± 8.0 (183)	48.5 ± 21.7 (262)
Cathepsin C	68 ± 2	83 ± 2	86 ± 5	32.6 ± 0	37.9 ± 65.2 (116)	97.4 ± 37.8 (299)
α-Iduronidase	73 ± 11	68 ± 13	71 ± 11	4.3 ± 0.5	5.0 ± 0.0 (116)	5.5 ± 1.2 (128)
α-Galactosidase	52 ± 8	56 ± 3	62 ± 3	6.7 ± 1.3	8.0 ± 0.4 (119)	7.3 ± 0.3 (109)
α-Galactosidase A	52 ± 10	57 ± 6	56 ± 7	5.9 ± 1.6	6.3 ± 0.3 (107)	7.4 ± 0.5 (125)
α-NAc-glucosaminidase	63 ± 10	71 ± 3	72 ± 5	0.4 ± 0.1	0.5 ± 0.3 (125)	1.2 ± 0.8 (300)
α-Fucosidase	52 ± 2	47 ± 8	46 ± 2	33.9 ± 5.7	31.8 ± 11.2 (94)	41.1 ± 3.7 (121)
β-Hexosaminidase A	58 ± 6	67 ± 7	66 ± 3	5.9 ± 1.3	8.0 ± 2.3 (136)	11.6 ± 3.9 (197)
Cathepsin B	0 ± 0	0 ± 0	1 ± 0	41.3 ± 2.3	64.5 ± 1.9 (156)	81.3 ± 24.8 (197)
Cathepsin L	60 ± 3	12 ± 1	31 ± 18	20.6 ± 2.8	47.6 ± 2.4 (231)	42.1 ± 12.4 (204)
Cathepsin D	44 ± 1	48 ± 9	45 ± 6	1069.6 ± 54.4	1492.4 ± 302.5 (139)	2111.4 ± 702.8 (197)
CLN2 protease	n.d.	n.d.	n.d.	22.3 ± 13.3	4.1 ± 0.4 (18)	53.7 ± 13.0 (241)
Thimet oligopeptidase	2 ± 2	3 ± 0	1 ± 2	28.9 ± 1.4	4.7 ± 0.9 (16)	12.5 ± 11.8 (43)
Acid phosphatase	1 ± 1	0 ± 0	1 ± 1	521.1 ± 152.9	560.6 ± 37.6 (108)	686.7 ± 151.9 (132)

overall increase in lysosomal enzyme levels because activities, on average, were elevated 1.9-fold compared with normal controls. In an earlier study, lysosomal enzyme activities in LINCL and JNCL were on average 1.5-fold and 2.2-fold higher than controls respectively [17]. Interestingly, the activity of the cytosolic thimet oligopeptidase was significantly decreased in LINCL.

Elevated Man-6-P glycoproteins in JNCL do not, in addition, seem to result from an increased fraction of enzymically active lysosomal enzymes represented by Man-6-P-containing forms. On average, the phosphorylated fraction of Man-6-P-containing lysosomal enzymes in JNCL was 1.1-fold greater than in the controls and in LINCL it was identical with controls. The only enzyme in which any alterations in phosphorylation were detected was cathepsin L, where the relative proportion of enzyme activity represented by the phosphorylated form was decreased to one-fifth and one-half of normal in LINCL and JNCL respectively.

DISCUSSION

In this paper we have demonstrated alterations in the levels of glycoproteins containing Man-6-P in three forms of NCL. In LINCL the most striking change was the absence of the CLN2 Man-6-P glycoprotein from patients compared with controls. Interestingly, however, another Man-6-P glycoprotein of slightly higher molecular mass seems to be elevated in LINCL. It is possible that this represents an increased synthesis of another lysosomal enzyme (perhaps in the same metabolic pathway as the CLN2 protein) as a cellular response to a lack of CLN2 activity and a subsequent accumulation of its substrate. Alternatively this band could represent a transient accumulation of a defective CLN2 precursor before its degradation.

Analysis of extracts from the adult form of NCL did not reveal any striking candidates for the protein underlying this disease, suggesting that it might be a rather less abundant lysosomal enzyme in brain than the CLN2 protein. However, it is clear that levels of the Man-6-phosphorylated form of two lysosomal enzymes (or two processing intermediates of the same enzyme) are elevated. This might also represent an attempt by diseased cells to compensate for the lack of an as yet unidentified lysosomal enzyme activity. If these Man-6-P glycoproteins participate in the same metabolic pathway as the actual defective protein in ANCL, their identification might provide valuable clues towards the molecular pathology of ANCL.

Man-6-P glycoproteins in JNCL were significantly elevated compared with those in controls; for most, this probably simply reflects increased synthesis of lysosomal enzymes. For several other Man-6-P glycoproteins, however, this seems not to be so. For example, levels of the band corresponding to the CLN2 protease were elevated 13-fold on average, even though the enzyme activity increased only 2.4-fold. This could reflect the accumulation of enzymically inactive Man-6-phosphorylated forms within diseased neurons, in which case much of the Man-6-phosphorylated proteins would not be measured with the enzymic assays (Table 2) but would be detectable by affinity blotting (Figures 1 and 2 and Table 1). Alternatively, changes in the carbohydrate structure of select lysosomal enzymes, resulting in more Man-6-P per protein molecule in JNCL, could result in an increased stoichiometry of receptor binding in the blotting assay.

In summary, we have detected increased levels of Man-6-P glycoproteins in JNCL that cannot simply be attributed to a generalized increased synthesis of lysosomal enzymes. Understanding the basis for these observations might well provide valuable clues to the cellular function of the CLN3 protein.

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