Phosphorylation of arylsulphatase A occurs through multiple interactions with the UDP-*N*-acetylglucosamine-1-phosphotransferase proximal and distal to its retrieval site by the KDEL receptor

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Phosphorylation of oligosaccharides of the lysosomal enzyme arylsulphatase A (ASA), which accumulate in the secretions of cells that mis-sort most of the newly synthesized lysosomal enzymes due to a deficiency of mannose 6-phosphate receptors, was found to be site specific. ASA residing within the secretory route of these cells contains about one third of the incorporated [²⁻³H]mannose in phosphorylated oligosaccharides. Oligosaccharides carrying two phosphate groups are almost 2-fold less frequent than those with one phosphate group and only a few of the phosphate groups are uncovered. Addition of a KDEL (Lys-Asp-Glu-Leu) retention signal prolongs the residence time of ASA within the secretory route 6-fold, but does not result in more efficient phosphorylation. In contrast, more than 90 % of the [²⁻³H]mannose incorporated into secreted ASA (with or without a KDEL retention signal) is present in phosphorylated

oligosaccharides. Those with two phosphate groups are almost twice as frequent as those with one phosphate group and most of the phosphate groups are uncovered. Thus, ASA receives *N*-acetylglucosamine 1-phosphate groups in a sequential manner at two or more sites located within the secretory route proximal and distal to the site where ASA is retrieved by the KDEL receptor, i.e. proximal to the *trans*-Golgi. At each of these sites up to two *N*-acetylglucosamine 1-phosphate groups can be added to a single oligosaccharide. Of several drugs known to inhibit transit of ASA through the secretory route only the ionophore monensin had a major inhibitory effect on phosphorylation, uncovering and sialylation.

Keywords: Golgi, lysosomal enzymes, mannose 6-phosphate.

INTRODUCTION

The majority of lysosomal enzymes are synthesized as soluble proteins in the endoplasmic reticulum (ER). Their trafficking to lysosomes depends on the generation of mannose 6-phosphate recognition markers, which allow the binding to mannose 6-phosphate receptors (MPRs) within the Golgi complex. The lysosomal enzyme-MPR complexes are packaged at the *trans*-Golgi network into clathrin-coated vesicles, which are ferried to and fuse with endosomal membranes. There the complexes dissociate. Whereas the lysosomal enzymes are routed to lysosomes, the receptors are transported back to the Golgi [1,2].

The mannose 6-phosphate recognition markers are generated by a sequence of reactions that starts with the transfer of *N*-acetylglucosamine 1-phosphate on to the hydroxy group of C-6 of the mannose residues in N-linked high-mannose oligosaccharides to generate phosphodiester. The diesters are then converted into the mannose 6-phosphate phosphomonoesters by the uncovering *N*-acetylglucosaminidase (EC 3.1.4.45), a complex of four identical type I membrane-spanning glycoprotein subunits [3]. The UDP-*N*-acetyl-*D*-glucosamine:lysosomal-enzyme *N*-acetylglucosaminephosphotransferase (EC 2.7.8.17) catalysing the first step is a multi-subunit enzyme composed of homodimers of three different subunits [4]. The enzyme recognizes a protein determinant on lysosomal enzymes, which is sensitive to denaturing conditions [5] and has been shown to depend on multiple lysine residues [6]. In cathepsin D, lysine

residues 203 and 293 are critical for its recognition by the phosphotransferase [6,7]. Additional sequences, however, are required for efficient phosphorylation of cathepsin D [8,9]. In a lysosomal enzyme polypeptide, generally more than one oligosaccharide is phosphorylated, and a single oligosaccharide can receive up to two phosphate groups. The multiple phosphorylation of a single oligosaccharide and the phosphorylation of more than one oligosaccharide per polypeptide chain raise the question of whether phosphorylation is the result of a single or multiple interactions between the phosphotransferase and its substrate. If separate recognition events occur, they may involve one or several recognition motifs on the polypeptide and one or a number of different phosphotransferases.

While the available data are compatible with the presence of either a single or multiple recognition motifs on a lysosomal enzyme [9], there is evidence that the biosynthesis of the mannose 6-phosphate recognition marker depends on more than a single interaction of phosphotransferase with a lysosomal enzyme polypeptide. This is based on the observation that inhibition of transport within the secretory route can lead to the accumulation of lysosomal enzyme precursors with oligosaccharides containing a single phosphodiester group. Upon release from transport block, phosphorylation is completed, yielding oligosaccharides that mostly carry two phosphomonoester groups [10].

In the present study we have analysed the phosphorylation of the lysosomal enzyme arylsulphatase A (ASA; EC 3.1.6.8). ASA carries three N-linked oligosaccharides, two of which are phos-

Abbreviations used: ASA, arylsulphatase A; AspN, endoprotease Asp-N; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; endo-H, endoglycosidase H; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblasts; MPR, mannose 6-phosphate receptors; wt, wild-type.

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phorylated [11]. We show that the phosphorylation pattern of the two glycosylation sites is site-specific. Furthermore, biosynthetic intermediates of ASA were analysed for their phosphorylation. This was facilitated by the use of cells that are deficient in MPRs. In these cells, essentially all newly synthesized lysosomal enzymes are en route between the endoplasmic reticulum (ER) and the plasma membrane [12]. Our findings support the view that oligosaccharides in ASA are phosphorylated in a stepwise fashion. The analysis of ASA with a KDEL (Lys-Asp-Glu-Leu) retention signal indicated that *N*-acetylglucosamine 1-phosphate groups are added proximal as well as distal to the KDEL-receptor retrieval site. The ionophore monensin, which is known to impair the passage of newly synthesized lysosomal enzymes through the secretory route, was found to inhibit the generation of the mannose 6-phosphate recognition marker.

EXPERIMENTAL

Construction of KDEL-ASA

cDNA of human ASA was subjected to site-directed *in vitro* mutagenesis (as described in [13]) using the non-coding oligonucleotide 5'-CCGAGGGGCCCTCCGGCCGGGGGATCTGGGCA-3' in order to generate a new *Eag*I site close to the stop codon. A second *Eag*I site at the 5'-end of the ASA cDNA was knocked out in a second round of *in vitro* mutagenesis without changing the amino acid sequence of ASA. Mutated cDNA of ASA was cloned into the expression vector pBEH and the resulting construct was digested with *Eag*I and *Sal*I. Oligonucleotides coding for the KDEL tetrapeptide (5'-GGCCAAAGATGAAGCTCTGAG-3' and 3'-TTTCTACTTGAGACTCAGCT-5') were phosphorylated at their 5'-ends, annealed and then ligated to the *Eag*I-*Sal*I-linearized vector pBEH-ASA. The presence of the mutation was confirmed by dideoxy DNA sequencing.

Cells and transfection

Immortalized mouse embryonic fibroblasts (MEFs) deficient in MPR 46 and MPR 300 (mpr⁻MEF; see [14]) were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal calf serum.

For stable transfection of mpr⁻MEF, human ASA-cDNA or cDNA for KDEL-ASA, cloned into the expression vector pBEH, was cotransfected with the selection marker pGKhygro [15] using the calcium phosphate precipitation technique [16]. Transfected cells were selected with an increasing concentration of hygromycin (Calbiochem-Novabiochem GmbH, Bad Soden, Germany) as described in [14]. Clones expressing wild-type ASA were identified by determination of the ASA activity in secretions [17]. The latter was 13-fold higher in the mpr⁻MEF/ASA clone used than in untransfected cells. KDEL-ASA-expressing clones were identified by metabolic labelling of cells with [³⁵S]methionine and subsequent immunoprecipitation of ASA-KDEL of cell lysates as described below.

Purification of [2-³H]mannose-labelled oligosaccharides of ASA and KDEL-ASA

mpr⁻MEF/ASA cells and mpr⁻MEF/KDEL-ASA cells were incubated in minimal essential medium containing 0.5 mM glucose and 5% (v/v) dialysed fetal calf serum for 1 h and then metabolically labelled with D-[2-³H]mannose (Amersham Life Science, Inc.; 666 GBq/mmol) for the time given in the legends of the Figures. Where indicated, a chase was performed by

adjusting the medium to 5 mM glucose in the absence or presence of 10 mM NH₄Cl, 10 μ M monensin, 0.1 mM chloroquine or 0.1 mM primaquine.

For a detailed analysis of the glycosylation-site-specific phosphorylation, ASA was affinity-purified from conditioned media and subjected to reductive carboxymethylation, proteolytic digestion with trypsin and endoproteinase Asp-N (AspN) and subsequent separation of peptides by reverse-phase HPLC as described previously [11]. Glycopeptides containing N-glycosylation sites I, II and III were isolated, digested with endoglycosidase H (endo-H), and free oligosaccharides were separated from endo-H-resistant glycopeptides by reverse-phase chromatography [18].

Tryptic peptides prepared from [³H]ASA secreted in the presence of drugs and controls were directly submitted to endo-H digestion without prior separation of peptides by HPLC. [³H]ASA from cells was isolated by immunoprecipitation [19], solubilized by heating for 5 min at 95 °C in 1% SDS, 10% glycerol, 12 mM dithiothreitol and 125 mM Tris/HCl, pH 6.8, and digested with endo-H [18]. For comparison of [³H]KDEL-ASA with [³H]wild-type-ASA ([³H]wt-ASA), both were isolated from cells and conditioned media by immunoprecipitation.

Analysis of anionic oligosaccharides on QAE-Sephadex

Oligosaccharides obtained after endo-H digestion were dissolved in 2 mM Tris-base, pH 9.5, and submitted to QAE-Sephadex anion-exchange chromatography using a linear gradient from 0 to 150 mM NaCl in 2 mM Tris-base as previously described [18]. Aliquots of gradient fractions were monitored for radioactivity and conductivity. For further analysis, peak fractions were pooled, lyophilized and desalted on a Sephadex G-10 column. Oligosaccharides derived from intracellular ASA were analysed by QAE-Sephadex chromatography using a step-wise gradient of 20, 60, 90 and 140 mM NaCl in 2 mM Tris-base.

Treatment of oligosaccharides with alkaline phosphatase and neuraminidase and mild acid hydrolysis

All steps were performed as previously described [18,20].

Metabolic labelling of cells and immunoprecipitation of ASA

mpr⁻MEF/ASA cells on 35 mm dishes were incubated in methionine-free medium containing 0.5 mM glucose for 1 h and then labelled with [³⁵S]methionine (Amersham Life Science, Inc.) in the same medium supplemented with 5% (v/v) dialysed fetal calf serum. During the subsequent chase period cells were incubated in complete medium (5 mM glucose) supplemented with 10 mM NH₄Cl, 10 μ M monensin, 0.1 mM chloroquine or 0.1 mM primaquine. Immunoprecipitation of ASA from cells and media was carried out with anti-(human placenta ASA) serum as described earlier [21]. Pellets were finally solubilized and subjected to SDS/PAGE and fluorography. Densitometric quantification of ASA was done using a Hewlett-Packard ScanJet 4c/T (Palo Alto, CA, U.S.A.) and the program WinCam2.2 (Microsoft).

RESULTS

Site-specific phosphorylation of ASA

ASA contains three N-glycosylation sites at Asn residues 158, 184 and 350, which are all utilized. Only oligosaccharides attached to glycosylation sites I (Asn-158) and III (Asn-350) are phosphorylated [11]. To characterize its phosphorylation, ASA was expressed in MEF lacking MPRs (mpr⁻MEF/ASA). These

Figure 1 Anion-exchange chromatography of ^3H -labelled oligosaccharides released by endo-H from N-glycosylation sites I, II and III of ASA

mpr⁻MEF expressing wt-ASA were labelled for 24 h with [$2\text{-}^3\text{H}$]mannose in medium containing 0.5 mM glucose. ASA was affinity purified from the medium. Glycopeptides representing N-glycosylation sites I, II and III were isolated as described in the Experimental section. They contained 45 (I), 18 (II) and 30 (III) % of the radioactivity associated with ASA. Oligosaccharides were released by endo-H and separated by QAE-Sephadex anion-exchange chromatography using a linear gradient from 0 to 150 mM NaCl in 2 mM Tris-base. The fractions (1 ml) were analysed for radioactivity (●) and conductivity (dotted line). Oligosaccharides carrying zero or 1–4 negative charges were pooled as indicated [the numbers below the bars refer to the fraction (%) of radioactivity present in the pool] and subjected to further analysis (see the Experimental section).

cells secrete more than 95 % of the newly synthesized ASA (see Figure 3). In contrast to intracellularly retained ASA, secreted ASA is not subjected to the secondary modifications of its oligosaccharide chains by glycosidases and phosphatases, which would occur after its transfer into endosomes/lysosomes. The oligosaccharides in ASA secreted by *mpr*⁻MEF reflect therefore the carbohydrate structure achieved in the biosynthetic route.

[^3H]ASA was purified from the secretions of cells grown in the presence of [$2\text{-}^3\text{H}$]mannose and 0.5 mM glucose. Glycopeptides carrying ASA glycosylation sites I, II and III were isolated by digestion with trypsin and endopeptidase AspN followed by reverse-phase HPLC [11]. The ^3H -labelled oligosaccharides were released from the glycopeptides by treatment with endo-H. Subsequent reverse-phase chromatography revealed that more than 85 % of the ^3H radioactivity associated with glycopeptides I–III was sensitive to endo-H. The ^3H -labelled oligosaccharides were separated by QAE-Sephadex anion-exchange chromatography at pH 9.5 into neutral oligosaccharides and those that carry one to four negative charges (Figure 1).

Whereas > 95 % of the oligosaccharides from glycosylation sites I and III carried two to four negative charges, 94 % of the oligosaccharides from glycosylation site II were neutral, the remainder carrying one negative charge, the nature of which was not analysed further (Figure 1). The individual anionic oligosaccharide fractions from sites I and III were further analysed by re-chromatography after mild acid hydrolysis and/or treatment with alkaline phosphatase and/or neuraminidase. This procedure allows one to differentiate oligosaccharides that are sialylated or phosphorylated or both, to distinguish oligosaccharides that carry one or two phosphate groups and to determine whether a phosphate group is present as a monoester or as a diester covered by an *N*-acetylglucosamine residue (see [20]). Mild acid hydrolysis converts the phosphodiester into a phosphomonoester and cleaves off neuraminic acid. Acidic oligosaccharides are thereby converted into neutral oligosaccharides (if they contain only neuraminic acid) and into species with two (monophosphorylated) or four (diphosphorylated) negative charges at pH 9.5. Treatment with alkaline phosphatase decreases the negative charge by two for every phosphate group removed, and treatment with neuraminidase reduces the negative charge by one for every neuraminic acid cleaved off.

The results for the oligosaccharides attached to N-glycosylation sites I and III of ASA are summarized in Table 1. About 93 % of oligosaccharides at site I are phosphorylated, carrying either one (20 %) or two (73 %) phosphate groups. About half of the oligosaccharides carrying one phosphate group are sialylated, suggesting that they are hybrid oligosaccharides, in which the α -1,3 branch of high-mannose oligosaccharides is replaced by a sialylated branch containing *N*-acetylglucosamine and galactose [22]. Moreover, most of the phosphate groups in these hybrid oligosaccharides are present as diesters. In monophosphorylated and diphosphorylated oligosaccharides of the high-mannose type the bulk of the phosphate groups (> 90 %) is uncovered.

At N-glycosylation site III, 88 % of the oligosaccharides are phosphorylated. Compared with site I, a slightly higher fraction (31 %) of the phosphorylated oligosaccharides is monophosphorylated and a higher fraction of the latter is sialylated ($\beta \sim 80$ %) and carries phosphodiester groups (≥ 60 %). Similar to site I, phosphate groups in diphosphorylated oligosaccharides are mostly (> 97 %) uncovered.

This analysis demonstrates that at each glycosylation site of ASA the phosphorylation pattern is distinct. At the two sites that are phosphorylated oligosaccharides with two phosphomonoesters represent the major species. Oligosaccharides of this type are known to mediate high-affinity binding to mannose 6-phosphate receptors [23–25].

Phosphorylation status of ASA in the secretory route

The phosphorylation of secreted ASA from *mpr*⁻MEF was compared with that of intracellular ASA. Since less than 5 % of the newly synthesized ASA is sorted in these cells to endosomes/lysosomes, ASA in *mpr*⁻MEF represents mostly ASA forms in transit through the secretory route.

ASA was isolated from cells after labelling with [$2\text{-}^3\text{H}$]mannose for 30 min, followed by a chase for up to 5 h in the presence of 5 mM glucose (Table 2). After being labelled for 30 min, ASA contains almost exclusively neutral oligosaccharides. Within 1 h of chase, acidic oligosaccharides with one, two or four negative charges appear. Their relative frequency remains rather constant throughout the chase of 5 h, comprising about one third of the oligosaccharides in ASA (shown in Figure 4A for the 2 h chase). Mild acid hydrolysis and treatment with alkaline phosphatase revealed that the oligosaccharides with one negative charge con-

Table 1 Characterization of endo-H sensitive oligosaccharides at N-glycosylation sites I and III of ASA

See Figure 1 for details of oligosaccharide fractions; —, not detected.

Oligosaccharide fraction	Frequency* (% per glycosylation site) of oligosaccharides						
	Neutral	Monophosphorylated		Diphosphorylated			Undefined†
		PM‡	PD‡	2PM	PM + PD	2PD	
I-2	—	9	9§	—	—	3	—
I-3	—	2§	—	3	—	3	—
I-4	—	—	—	67	—	—	—
Fraction I total	4	20	—	—	73	—	3
III-2	—	6	18§	—	—	—	—
III-3	—	7§	—	—	3	—	8
III-4	—	—	—	54	—	—	—
Fraction III total	4	31	—	—	57	—	8

* The frequency refers to the fraction of ^3H radioactivity present in the oligosaccharide fraction. Since the number of $[2\text{-}^3\text{H}]$ mannose residues per oligosaccharide can vary among the different oligosaccharides, the fraction of ^3H radioactivity may differ from the molar fraction.

† Refers to non-phosphorylated oligosaccharides, which were converted by mild acid hydrolysis to neutral species.

‡ PM, phosphomonoester; PD, phosphodiester.

§ Refers to sialylated oligosaccharides. For fraction I-3 and III-3 the analysis did not permit differentiation between oligosaccharides carrying one phosphomonoester and one sialic acid residue and oligosaccharides carrying one phosphodiester and two sialic acid residues.

tained one phosphodiester group, oligosaccharides with two negative charges contained two phosphodiesters and oligosaccharides with four negative charges contained two phosphomonoesters. Diphosphorylated species are 1.6–3.2 times less frequent than monophosphorylated species (ratio 0.26–0.64). Furthermore, uncovered phosphate groups represent only a minor fraction and are found only in diphosphorylated oligosaccharides.

In contrast to intracellular ASA, the bulk of ^3H -labelled oligosaccharides in ASA secreted during the 5 h chase are phosphorylated. Diphosphorylated species are almost twice as frequent as monophosphorylated species (ratio of 1.7) and less than one third of the phosphate groups are covered (see Tables 3 and 4 and Figure 4B). It should be noted that the fraction of radioactivity in neutral oligosaccharides and the frequency of diphosphorylated versus monophosphorylated oligosaccharides were lower when the ASA was from cells grown in the presence of 5 mM glucose (Table 3) rather than 0.5 mM glucose (Table 1 and Figure 1).

A parallel analysis was performed with KDEL-ASA expressed in mpr[−]MEF. Addition of the KDEL tetrapeptide sequence to

Table 3 Effect of NH_4Cl , primaquine, chloroquine and monensin on the phosphorylation of ^3H -labelled oligosaccharides in secreted ASA

Addition during the chase	Frequency of unphosphorylated oligosaccharides (% of total endo-H sensitive oligosaccharides)	Ratio of di- versus mono-phosphorylated oligosaccharides
—	12	1.7
NH_4Cl	18	2.4
Primaquine	9	1.8
Chloroquine	12	1.5
Monensin	29	1.8

the C-terminus of ASA was expected to allow retention of ASA in the secretory route through binding to the KDEL receptor and recycling to the ER. Since the bulk of KDEL receptor is located in the *cis*-Golgi, KDEL-ASA should be enriched in forms that have not encountered the oligosaccharide modifying enzymes located in the mid- and *trans*- parts of the Golgi complex.

Table 2 Charge distribution of endo-H-sensitive oligosaccharides from intracellular (A) and secreted (B) wt-ASA and KDEL-ASA

Results are given as radioactivity (% of total) in endo-H-sensitive oligosaccharides with the number of negative charges indicated. —, not detectable.

Chase (h)	Negative charge(s)...	Radioactivity (% of total)									
		wt-ASA					KDEL-ASA				
		0	1	2	3	4	0	1	2	3	4
A —		96	3	—	—	—	98	2	—	—	—
1		75	19	4	—	2	83	15	2	—	—
2		66	27	5	—	2	65	28	6	—	—
5		64	22	7	—	7	56	29	14	—	—
B 5		7	6	23	10	54	2	2	16	17	63

Figure 2 Secretion of wt-ASA and of KDEL-ASA

mp^r-MEF expressing wt-ASA (○) or KDEL-ASA (●) were labelled for 15 min with [³⁵S]methionine and then chased for up to 165 min in complete medium. ASA was immunoprecipitated from extracts of cells and media and separated by SDS/PAGE (upper part). The lower diagram gives the percentage of secreted [³⁵S]ASA as calculated by densitometry of the fluorograms.

Secretion of KDEL-ASA was indeed greatly retarded. Whereas half of newly synthesized wild-type ASA is secreted within 2 h of chase, the amount of secreted KDEL-ASA after 2 h is still below the limit of detection (Figure 2). When the chase was prolonged to up to 13 h eventually half of the KDEL-ASA was secreted (results not shown).

The analysis of ³H-labelled oligosaccharides derived from intracellular KDEL-ASA (Table 2) revealed a phosphorylation pattern similar to that of wt-ASA. A difference was that even after a 5 h chase oligosaccharides with two phosphomonoesters were below the limit of detection in KDEL-ASA. In addition, phosphorylation was slightly retarded. The small amount of KDEL-ASA secreted during 5 h of chase contained oligosaccharides with two phosphomonoester groups as the most abundant species (60% of all oligosaccharides), indicating that oligosaccharides in KDEL-ASA escaping retrieval to the ER become phosphorylated and uncovered in the more distal parts of the Golgi as in wt-ASA.

Effect of weak bases and monensin on the phosphorylation of ASA

Proton gradient-disturbing agents such as weak bases and the ionophore monensin are known to retard the transport of lysosomal enzymes within the secretory route and to impair their sorting [26]. In addition, uncovering of phosphate groups was found to be reduced in human skin fibroblasts treated with 10 mM NH₄Cl [27].

We used mp^r-MEF overexpressing ASA to examine whether weak bases such as NH₄Cl, chloroquine, primaquine and the ionophore monensin affect the processing of phosphorylated oligosaccharides in lysosomal enzymes. When cells were labelled for 30 min with [³⁵S]methionine and then chased for 6 h about

Figure 3 Effect of NH₄Cl, monensin, primaquine and chloroquine on secretion of ASA

Cells were labelled for 30 min with [³⁵S]methionine and then chased for 6 h with complete medium supplemented with 10 mM NH₄Cl, 10 μM monensin, 0.1 mM primaquine or 0.1 mM chloroquine. ASA was immunoprecipitated from extracts of cells (C) and media (M), separated by SDS/PAGE and quantified by densitometry of the fluorogram. The numbers below the lanes represent the percentage of [³⁵S]ASA recovered in the secretions.

98% of the newly synthesized ASA was recovered in the secretions. The presence of 10 mM NH₄Cl, 0.1 mM primaquine or 0.1 mM chloroquine during the chase reduced the secretion to 51–59%, whereas 10 μM monensin reduced secretion to 17% (Figure 3).

ASA was isolated from secretions of cells that had been labelled for 30 min with [2-³H]mannose and then chased for 5 h in the absence or presence of the drugs. The relative frequency of neutral oligosaccharides, the ratio of di- versus mono-phosphorylated oligosaccharides (Table 3) and the uncovering of diphosphorylated oligosaccharides (results not shown) were similar in ASA from control cells and cells treated with primaquine or chloroquine. Treatment with NH₄Cl had moderate effects on the ratio of di- versus mono-phosphorylated oligosaccharides (increase from 1.7 to 2.4, see Table 3) and the uncovering of diphosphorylated oligosaccharides (decrease from 95% uncovering in controls to 81%, results not shown).

Quantitatively more important effects were seen in ASA secreted by cells treated with monensin (Table 3 and Figure 4D). The fraction of neutral oligosaccharides increased to 29%. The ratio of di- versus mono-phosphorylated oligosaccharides was normal, but uncovering of diphosphorylated oligosaccharides decreased. Furthermore, sialylation was completely inhibited in the presence of monensin (Table 4).

We also examined the phosphorylation of intracellular ASA from cells chased in the presence of NH₄Cl, primaquine and monensin. If transport of ASA is inhibited at a specific site of the secretory route, intracellular ASA should be enriched in forms with a phosphorylation pattern characteristic of compartments proximal to the transport block at the expense of forms that are generated in compartments distal to the block. Cells were labelled for 30 min with [2-³H]mannose and then chased for 2 h in the absence or presence of the drugs. Effects of NH₄Cl, primaquine or monensin were only noted for the ratio of di- versus mono-phosphorylated oligosaccharides, which increased moderately from 0.3 in controls to 0.5 and 0.7 in the presence of NH₄Cl and primaquine respectively, and more markedly to 1.3 in the presence of monensin. Moreover, almost 50% of the phosphate groups in diphosphorylated oligosaccharides were uncovered (Figure 4C).

DISCUSSION

Site-specific phosphorylation of ASA

Phosphorylation of ASA is known to be restricted to the oligosaccharides attached to Asn-158 and Asn-350 [11]. The phosphorylation patterns at both sites resemble each other in

Figure 4 Anion-exchange chromatography of ^3H -labelled oligosaccharides released by endo-H from intracellular and secreted ASA from control and monensin-treated cells

Cells were labelled for 30 min with [^3H]mannose and then chased for 2 h or 5 h with complete medium without (**A** and **B**) or supplemented with (**C** and **D**) 10 μM monensin. ASA was isolated from cells after a 2 h chase (**A** and **C**) and from secretions after a 5 h chase (**B** and **D**). Oligosaccharides were released from ASA by endo-H and separated by anion-exchange chromatography using a stepwise (**A** and **C**) or linear (**B** and **D**) gradient of NaCl in 2 mM Tris-base.

Table 4 Effect of monensin on oligosaccharides in secreted ASA

The oligosaccharides were pooled as shown in Figures 4(B) and 4(D) and subjected to mild acid hydrolysis and treatment with alkaline phosphatase and/or neuraminidase.

Oligosaccharide fraction	Addition during chase	Frequency (% of total oligosaccharides)	Uncovered phosphate groups (% of phosphate groups present in the oligosaccharide fraction)	Sialylated oligosaccharides (% of total oligosaccharides)
Non-phosphorylated oligosaccharides	—	10	—	3
	Monensin	29	—	—
Monophosphorylated oligosaccharides	—	33	51	15
	Monensin	25	56	—
Diphosphorylated oligosaccharides	—	57	94	—
	Monensin	46	64	—

that the majority of oligosaccharides carry two phosphomonoesters and that a substantial fraction of monophosphorylated oligosaccharides retain the phosphate group as a phosphodiester and contain sialic acid. The latter indicates that these oligosaccharides are of the hybrid type, with the phosphate group attached to the α -1,6 branch, while the α -1,3 branch is replaced by a complex-type branch containing *N*-acetylglucosamine and galactose. The fraction of oligosaccharides carrying a single phosphate group, the degree of uncovering and the sialylation of the monophosphorylated oligosaccharides, however, were distinct for the two sites.

Site-specific patterns of phosphorylation are commonly found in lysosomal enzymes carrying multiple N-linked oligosaccharides [28–31]. Similar to ASA, human cathepsin D [32,33] and bovine DNase I [34] are phosphorylated at two sites. In both enzymes, oligosaccharides carrying two phosphate groups are restricted to one of two sites. Apparently, only some of the oligosaccharides are positioned at the surface of lysosomal enzymes such as ASA, cathepsin D and DNase I in a manner

such that both the α -1,6 and the α -1,3 branch can be phosphorylated.

Stepwise generation of the mannose 6-phosphate recognition marker

The majority of phosphorylated oligosaccharides in ASA transiting the secretory route contain a single phosphodiester group, whereas after passing the secretory route the majority of phosphorylated oligosaccharides carry two phosphomonoester groups. This clearly indicates that the two *N*-acetylglucosamine 1-phosphate groups are added in a sequential manner and also that uncovering by the α -*N*-acetylglucosaminidase is separated in time, and presumably also in space, from the phosphorylation.

The ER retention signal KDEL was added to the C-terminus of ASA to refine the sites where phosphorylation and uncovering occur. Addition of the retention signal prolonged the mean residence time of ASA within the secretory route approx. 6-fold from 2 h to about 13 h. The retention is likely to involve retrieval

of ASA exiting the ER by binding to KDEL receptors in post-ER compartments, retrograde transport of the KDEL receptor-ASA complexes and release of ASA in the ER [35]. The KDEL receptor is found throughout the Golgi and its capacity to retrieve ligands extends to the *trans*-Golgi network [36]. However, the bulk of ligands appear to become retrieved from sites much closer to the exit site at the ER, e.g. from the *cis*-Golgi or even more proximal [37].

Retention of ASA did not increase the fraction of phosphorylated oligosaccharides. Among the phosphorylated oligosaccharides, those carrying two phosphate groups were slightly decreased, and uncovering of phosphate groups or sialylation were even below the limit of detection. Thus phosphotransferases residing within the proximal part of the secretory route, up to the site where the ligands bind to the KDEL receptor, can add up to two *N*-acetylglucosamine 1-phosphate groups per oligosaccharide. The failure to obtain a more extensive phosphorylation when the residence time of ASA within this compartment is prolonged strongly indicates that the properties of the phosphotransferases and/or the accepting ASA limit the phosphorylation. Addition of the remaining *N*-acetylglucosamine 1-phosphate groups requires that ASA traverses the boundary where KDEL receptors retrieve their ligands. The fractions of *N*-acetylglucosamine 1-phosphate groups added to ASA proximal and distal to this boundary are about equal. Distal to this boundary are also located the uncovering α -*N*-acetylglucosaminidase and the sialyltransferases. This implies that this boundary is proximal to *trans*-Golgi network.

When the transport within the secretory route is inhibited by low temperature or the ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), only oligosaccharides with a single *N*-acetylglucosamine 1-phosphate group are synthesized. Phosphorylation and uncovering are completed upon reversal of the transport inhibition, e.g. by raising the temperature [10]. The most likely explanation for this observation is that phosphorylation occurs in at least two steps located proximal and distal to the site of transport inhibition. Our results, obtained with wt-ASA and KDEL-ASA transiting the secretory route under physiological conditions, eliminate the possibility that in this earlier study low temperature and CCCP had artificially induced phosphorylation at two sites by redistributing the phosphotransferases.

The phosphotransferases interacting with ASA along the secretory route may be differently composed and thereby exhibit different enzymic properties. This would explain why prolonged residence within the early section of the secretory route does not result in a more complete phosphorylation, despite the fact that the phosphotransferase present in this section can add up to two *N*-acetylglucosamine 1-phosphate groups to a single oligosaccharide. Addition of the second *N*-acetylglucosamine 1-phosphate is known to depend on prior removal of at least one α -1,2-linked mannose from the α -1,6 branch [38]. The presence of diphosphorylated oligosaccharides indicates that ASA is exposed within this early section of the secretory route not only to the phosphotransferases but also to α -mannosidases [39].

At each phosphorylation site the phosphotransferases may interact with different recognition signals on the surface of lysosomal enzymes. For cathepsin D, cathepsin L and DNase I a major phosphorylation signal involving two lysine residues about 34 Å apart has been characterized [6]. In addition, weaker sites for lysosomal enzyme-phosphotransferase interaction may exist [6]. Clearly, further studies, including the reconstitution of lysosomal enzyme phosphorylation with purified components, are required for an understanding of the biogenesis of the mannose 6-phosphate recognition marker.

Effect of weak bases and of monensin on transport and phosphorylation of lysosomal enzymes

Under conditions where the weak bases NH_4Cl , chloroquine and primaquine impair lysosomal enzyme transport within the secretory route with comparable efficiency, only NH_4Cl has a slight inhibitory effect on the uncovering of α -*N*-acetylglucosaminidase. This is in agreement with an earlier study [27]. Weak bases did not increase the fraction of phosphorylated oligosaccharides in ASA, suggesting that they inhibit transport of lysosomal enzymes within the proximal section of the secretory pathway as defined by the KDEL receptor.

The sodium ionophore monensin inhibits terminal glycosylation of many glycoproteins and this effect is generally explained by inhibition of transport to the *trans*-Golgi, where the responsible glycosyltransferases reside [40]. Monensin, which, like weak bases, inhibits trafficking and sorting of lysosomal enzymes [26,41], induces the accumulation of polypeptides with diphosphorylated oligosaccharides, with a high degree of uncovering. This defines one site of the transport block as being distal to the site where terminal phosphorylation and uncovering of phosphodiesterases occurs. Moreover, the decrease in phosphate groups and of their uncovering, as well as the absence of sialylated (hybrid type) phosphorylated oligosaccharides in secreted ASA, point to an inhibitory effect of monensin on these steps, either by inhibiting the respective enzymes or by allowing ASA to bypass these enzymes.

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