Carbohydrate Research, 197 (1990) 217–226
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

EFFECT OF GLYCOSYLATION INHIBITORS AND ACIDOTROPIC AMINES ON THE SYNTHESIS, PROCESSING, AND INTRACELLULAR–EXTRACELLULAR DISTRIBUTION OF α-L-FUCOSIDASE IN B-LYMPHOBLASTOID CELLS*

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(Received December 27th, 1988; accepted for publication in revised form, May 18th, 1989)

ABSTRACT

N-Methyldeoxynojirimycin, 1-deoxymannojirimycin, and monensin interferred with normal processing of asparagine-linked oligosaccharide chains of α-L-fucosidase in lymphoid cells by blocking conversion of high-mannose oligosaccharides of newly made precursor enzyme to complex oligosaccharides of mature intracellular and extracellular forms of enzyme. These compounds did not substantially alter the distribution of newly made α-L-fucosidase between intracellular and extracellular compartments. Thus, sorting of newly made α-L-fucosidase molecules that are retained intracellularly from molecules that are eventually secreted does not require terminal glycosylation or the trimming of glucose or α-D-(1→2)-linked mannose residues from carbohydrate chains. Chloroquine and ammonium chloride had no substantial effect on the structural processing or on the intracellular–extracellular distribution of α-L-fucosidase in lymphoid cells. In other cell types, these weak bases caused a massive secretion and an intracellular deficiency of acid hydrolases. The different responses to weak bases in lymphoid cells and the other cell types can be explained either by an inability of these agents to neutralize the pH of intracellular organelles in lymphoid cells or by a routing mechanism in lymphoid cells that is independent of pH.

INTRODUCTION

α-L-Fucosidase (α-L-fucoside fucohydrolase, EC 3.2.1.51) is an acid hydrolase that has been ubiquitously found in human tissues and extracellular fluids with cellular enzyme located in lysosomes[1]. In cultured lymphoid cells, α-L-fucosidase is synthesized as a precursor form with a M_r 58 000 that is processed to either mature intracellular or extracellular forms having M_r 60 000 and 62 000, respectively[2]. All three enzyme forms are glycoproteins with a common polypeptide

*This research was supported by Grant DK 32161 from the National Insitutes of Health.

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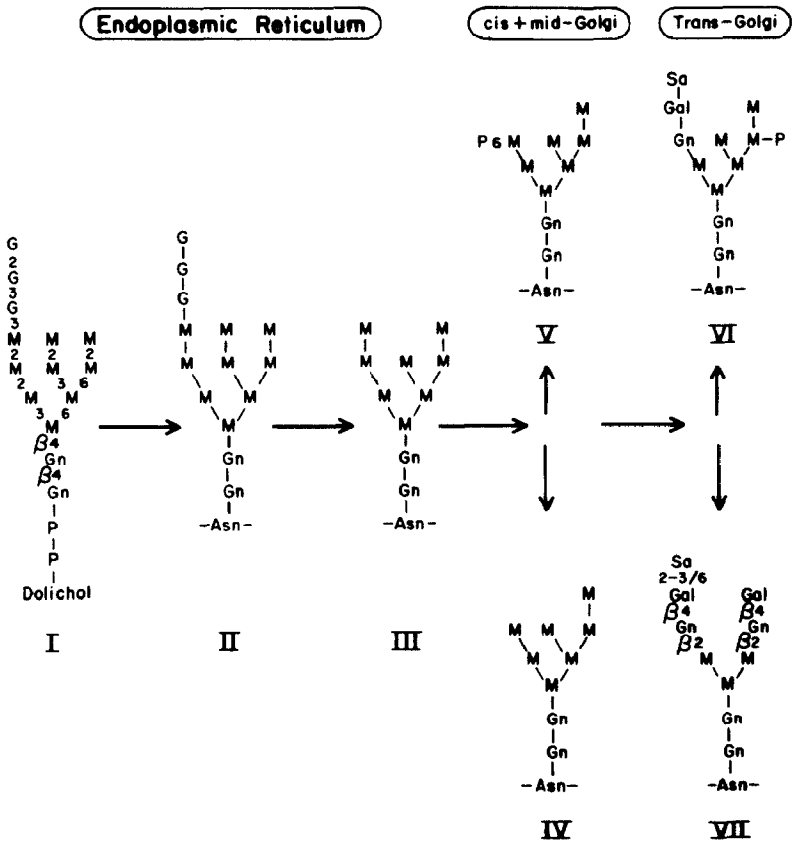

Scheme 1. Main stages in the processing of oligosaccharides in lysosomal acid hydrolases. Modified from ref. 5. The organelles to which processing is localized are indicated at the top. Symbols represent: (G) D-glucose, (Gal) D-galactose, (Gn) 2-acetamido-2-deoxy-D-glucose, (M) D-mannose, (Sa) *N*-acetylneuraminic acid, and (P) phosphate. Single numbers indicate the positions of α-D-anomerically-linked sugars. Four typical structures that have been found in lysosomal acid hydrolases as formed in the Golgi complex are: (IV) high-mannose, (V) phosphorylated high-mannose, (VI) phosphorylated hybrid, and (VII) complex carbohydrate. Structures II, III, IV, V, and VI are sensitive to hydrolysis by endo-H but structure VII is not. Tunicamycin prevents formation of I. *N*-Methyldeoxynojirimycin inhibits the removal of the three D-glucose residues from II. 1-Deoxymannojirimycin inhibits the removal of (1→2)-linked-α-D mannose residues from II and III. Monensin prevents the addition of 2-acetamido-2-deoxy-D-glucose, D-galactose, and *N*-acetylneuraminic acid to VI and VII. Ammonium chloride and chloroquine disrupt normal transport of acid hydrolases bearing structures V and VI to lysosomes. This results in an intracellular deficiency and massive secretion of acid hydrolases.

chain of M_r 52 000 but with a different carbohydrate component. The carbohydrate component of the intracellular, precursor form is completely sensitive to hydrolysis by endo-*N*-acetyl-β-D-glucosaminidase H (endo-H). However, the carbohydrate component of the majority of the intracellular, mature molecules, and all of the extracellular, mature form is completely resistant. Endo-H catalyzes the hydrolysis of the di-*N*-acetylchitobiose residue of *N*-glycosyl-linked, high-mannose chains and some hybrid chains of glycoproteins[3]. The enzyme does not cleave complex-carbo-

hydrate chains. Therefore, the precursor form of α-L-fucosidase probably contains high-mannose structures that are converted to mature forms with complex-carbohydrate structures. It has been reported that processing of carbohydrate chains of acid hydrolases involves formation of endo-H-sensitive structures having D-mannosyl phosphate residues, which are required for transport of acid hydrolases from the Golgi apparatus to lysosomes *via* D-mannosyl phosphate receptors[4–6] (Scheme 1). Since the carbohydrate component of the majority of mature, intracellular α-L-fucosidase molecules in lymphoid cells is completely resistant to endo-H, routing of these α-L-fucosidase molecules may involve alternative structural requirements. This hypothesis has additional support from the study of I-cell disease. Cells from patients with I-cell disease are unable to synthesize the D-mannosyl phosphate recognition marker of lysosomal enzymes because of an inherited deficiency of *N*-acetylglucosaminyl phosphotransferase[5,6]. Consequently, acid hydrolases cannot bind to D-mannosyl phosphate receptors. In fibroblasts, this results in a massive secretion of acid hydrolases and an intracellular deficiency of these enzymes. However, in other cell types, including lymphoid cells, there are normal levels of intracellular acid hydrolases, including α-L-fucosidase, despite the absence of *N*-acetylglucosaminyl phosphotransferase[5–7].

Glycosylation inhibitors and acidotropic amines have been shown to block critical steps in the processing and routing of acid hydrolases (Scheme 1) and have proved useful for understanding trafficking of acid hydrolases in fibroblasts, hepatocytes, macrophages, and monocytes[4,5,8–16]. In this report, we have surveyed the effects of these agents on the processing and intracellular–extracellular distribution of newly synthesized α-L-fucosidase in lymphoid cells to gain additional information on the requirements for routing of α-L-fucosidase.

EXPERIMENTAL

Materials. — Tunicamycin, monensin, and chloroquine were obtained from Sigma, NH_4Cl was obtained from EM Science (Cherry Hill, NJ), and *N*-methyldeoxynojirimycin, 1-deoxymannojirimycin, and endo-H were obtained from Genzyme (Boston, MA).

Cell culture. — The establishment, growth conditions, and background information of human B-lymphoblastoid cell line, B142, have been published[2].

Immunoprecipitation of α-L-fucosidase. — Procedures have been described for (a) radiolabeling of cultured cells with [^{35}S]methionine, (b) preparation of cell and medium extracts, (c) measurement of incorporation of [^{35}S]methionine into acid-precipitable total protein of extracts, (d) immunoprecipitation of α-L-fucosidase from extracts, (e) gel electrophoresis of immunoprecipitates under denaturing and reducing conditions, (f) visualization of gels by fluorography, and (g) quantitation of radioactive bands of gels[2]. In some experiments, immunoprecipitates were digested with endo-H before gel electrophoresis as previously published[2].

RESULTS

Tunicamycin inhibits asparagine-linked glycosylation by blocking the first addition of carbohydrate to the polypeptide chain of putative glycoproteins[10] (see Scheme 1). To determine whether prevention of asparagine-linked glycosylation interferes with intracellular–extracellular routing of α-L-fucosidase in lymphoid cells, cultures were treated with tunicamycin (Figs. 1 and 2). Cells pulse-labeled with [^{35}S]methionine for 1.5 h in the presence of tunicamycin (10 μg/mL) had a major form of α-L-fucosidase with a M_r 52 000. Control cultures not incubated with tunicamycin had a major form having M_r 58 000. This suggested that α-L-fucosidase has an unglycosylated polypeptide chain of M_r 52 000, which agrees with other structural studies[2]. Cells pulse-labeled for 1.5 h and chased for 21 h with unlabeled methionine in the presence of tunicamycin (10 μg/mL) had an intracellular form having M_r 52 000 and a major extracellular form having M_r 54 000. After the pulse-chase, control cultures not treated with tunicamycin had intracellular and extracellular forms of α-L-fucosidase with molecular masses ranging between 60 000 and 62 000. The increase in molecular mass of α-L-fucosidase in control cultures that happens between 1.5 h and 21 h was due to processing of oligosaccharide components from high-mannose to complex-carbohydrate chains[2]. The increase in molecular mass of extracellular α-L-fucosidase in tunicamycin (10 μg/mL)-treated cultures probably reflected limited glycosylation. In the pulse and pulse-chase experiments (Figs. 1 and 2), cultures treated with tunicamycin (10 μg/mL) had decreased amounts of total (intracellular plus extracellular) immunoprecipitable α-L-fucosidase protein relative to untreated cultures, as well as decreased amounts

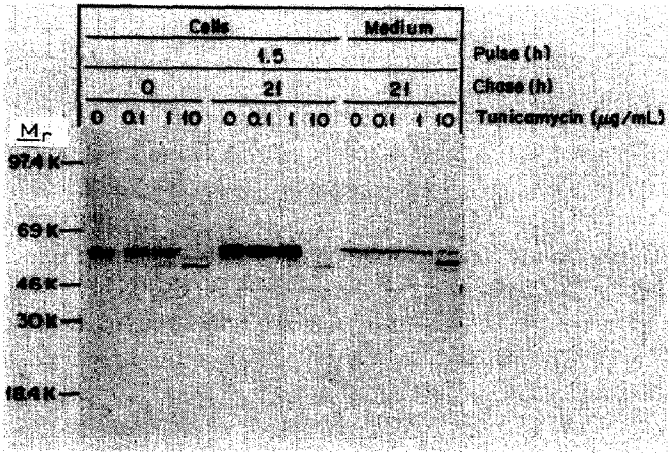

Fig. 1. Effect of tunicamycin on the processing of α-L-fucosidase in B142 cells. Cells were preincubated with or without tunicamycin for 1.5 h before either a 1.5-h pulse-labeling with [^{35}S]methionine or a 1.5-h pulse and a 21-h chase with unlabeled methionine. Tunicamycin was present throughout the pulse-chase period. α-L-Fucosidase was isolated by immunoprecipitation, and then subjected to electrophoresis, detection, and quantitative determination as described in the Experimental section.

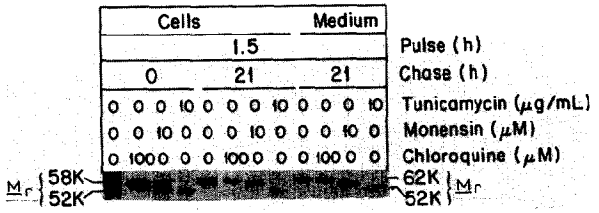

Fig. 2. Effect of tunicamycin, monensin, or chloroquine on the processing of α-L-fucosidase in B142 cells. Cells were preincubated with either tunicamycin, monensin, or chloroquine for 1.5 h before either a 1.5-h pulse-labeling with [^{35}S]methionine or a 1.5-h pulse and a 21-h chase with unlabeled methionine. Either tunicamycin, monensin, or chloroquine was present throughout the pulse-chase period. α-L-Fucosidase was isolated by immunoprecipitation and then subjected to electrophoresis, detection, and quantitative determination as described in the Experimental section.

of acid-precipitable protein labeled with [^{35}S]methionine (data not shown). This demonstrated that tunicamycin increased the turnover of both α-L-fucosidase and general protein. Thus, the effects of tunicamycin are complex and preclude a simple evaluation of structural requirements for routing of α-L-fucosidase.

N-Methyldeoxynojirimycin obstructs the normal processing of asparagine-linked oligosaccharides of glycoproteins by inhibiting the action of glucosidases that remove the three D-glucose residues from nascent oligosaccharides[10,13] (see Scheme 1). To determine if interference with structural processing of asparagine-linked carbohydrate chains alters intracellular–extracellular routing of α-L-fucosidase in lymphoid cells, triplicate cultures of B142 cells were treated without or with *N*-methyldeoxynojirimycin (0.3 and 1.0mM). In its presence, cells pulse-labeled for 1.5 h with [^{35}S]methionine or pulse-labeled for 1.5 h, followed by a 21-h chase with methionine exhibited a dose-dependent increase of 1000–2000 in the molecular mass of intracellular α-L-fucosidase (data not shown). It was published that *N*-methyldeoxynojirimycin causes similar increases in molecular mass of glycoproteins in hepatocytes[17]. This was ascribed to the presence of D-glucose residues in carbohydrate chains. If processing of α-L-fucosidase is blocked at the stage of removal of D-glucose residues, the carbohydrate component of α-L-fucosidase would not be converted from an endo-H-sensitive to an endo-H-resistant form. Indeed, the processing of the carbohydrate component of α-L-fucosidase from endo-H sensitive to resistant forms was blocked by *N*-methyldeoxynojirimycin (Fig. 3A), suggesting that this compound prevents formation of complex-carbohydrate chains, presumably by blocking removal of D-glucose residues. In cells pulse-labeled for 1.5 h with [^{35}S]methionine, incorporation of radiolabel into immunoprecipitable α-L-fucosidase was reduced about two-fold in cultures treated with mM *N*-methyldeoxynojirimycin, but incorporation of radiolabel into acid-precitable protein was not affected (data not shown). This suggested that α-L-fucosidase synthesis was inhibited but not general protein synthesis. In cells pulse-labeled for 1.5 h with [^{35}S]methionine and chased for 21 h with unlabeled methionine, the percentage of total (intracellular and extracellular) immunoprecipitable α-L-

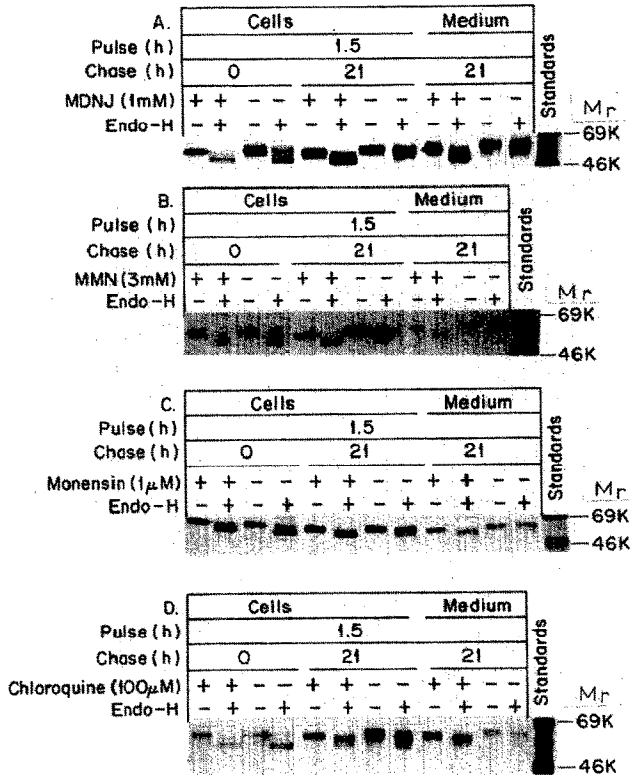

Fig. 3. Effect of endo-H on α-L-fucosidase immunoprecipitated from B142 cells treated with either: (A) *N*-methyldeoxynojirimycin (MDNJ), (B) 1-deoxymannojirimycin (MMMN), (C) monensin, or (D) chloroquine. α-L-Fucosidase was isolated by immunoprecipitation and divided into two portions. One portion was incubated with endo-H and the other served as a control without endo-H. After incubation, each sample was subjected to electrophoresis, detection, and quantitative determination as described in the Experimental section. In Fig. 3C, a slight curvature at the top of the poly(acrylamide) gel caused the bow-shaped migration of the samples.

fucosidase found extracellularly for the culture treated with mM *N*-methyldeoxynojirimycin and for the untreated control culture were virtually identical (data not shown). These results suggested that prevention of processing of asparagine-linked carbohydrate chains by *N*-methyldeoxynojirimycin reduced synthesis of α-L-fucosidase, but did not alter the distribution of α-L-fucosidase between intracellular and extracellular compartments.

1-Deoxymannojirimycin and monensin interfere with the normal processing of asparagine-linked oligosaccharides of glycoproteins. 1-Deoxymannonojirimycin inhibits the action of D-mannosidases that remove (1→2)-linked α-D-mannose residues from nascent oligosaccharides of glycoproteins[10,13] (see Scheme 1). This prevents terminal glycosylation of high-mannose structures to complex-carbohydrate structures. Monensin is a monovalent ionophore that impairs conversion of high-mannose chains to complex-carbohydrate chains by disrupting the integrity of

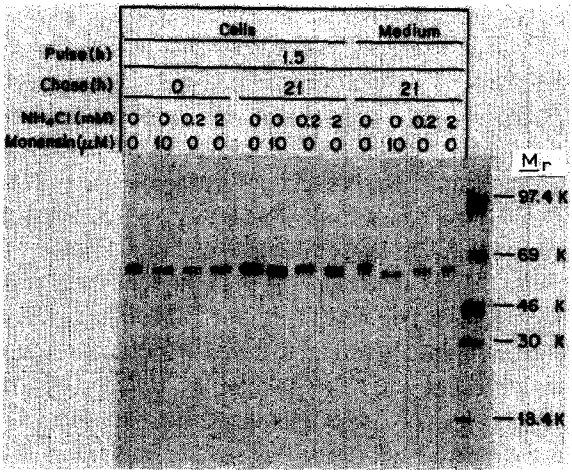

Fig. 4. Effect of ammonium chloride or monensin on processing of α-L-fucosidase in B142 cells. Cultures were pulse-labeled for 1.5 h with [^{35}S]methionine or pulse-labeled for 1.5 h and chased for 21 h with unlabeled methionine in the presence of ammonium chloride or monensin. α-L-Fucosidase was isolated by immunoprecipitation and then subjected to electrophoresis, detection, and quantitative determination as described in the Experimental section.

the Golgi complex where terminal glycosylation normally occurs[11] (see Scheme 1). To determine whether blocking terminal glycosylation of asparagine-linked carbohydrate chains disrupts the intracellular–extracellular routing of α-L-fucosidase in lymphoid cells, replicate cultures were treated without or with either 1-deoxymannojirimycin (0.3, 1.0, and 3.0mM) or monensin (0.1, 1.0, and 10μM). In pulse-chase experiments, both 1-deoxymannojirimycin (3.0mM) and monensin (0.1–10 μM) blocked conversion of the intracellular precursor form of α-L-fucosidase having M_r 58 000 into the intracellular and extracellular mature forms with M_r 60 000 and 62 000, respectively (Figs. 2 and 4 are representative). Instead, cultures treated with 1-deoxymannojirimycin (3mM) or monensin had intracellular and extracellular enzyme forms having M_r 58 000. Concomitantly, these compounds prevented processing of α-L-fucosidase from an endo-H-sensitive precursor form to endo-H-resistant mature forms (Figs. 3B and 3C). These data indicated that both compounds inhibited terminal glycosylation of α-L-fucosidase. In cells pulse-labeled for 1.5 h with [^{35}S]methionine and chased for 21 h with unlabeled methionine (Fig. 3B), the percentage of total (intracellular plus extracellular) immunoprecipitable α-L-fucosidase found extracellularly for cultures treated with 3mM 1-deoxymannojirimycin and for untreated control cultures were 20 and 22%, respectively. In analogous pulse-chase experiments (Figs. 2 and 4), the average percentages of total α-L-fucosidase found extracellularly for cultures treated with 10μM monensin and for untreated control cultures were 37 and 38%, respectively. Additionally, in cells pulse-labeled for 1.5 h with ^{35}S-methionine (Figs. 2, 3B, and 4), incorporation of radiolabel into immunoprecitable α-L-fucosidase or into acid-precipitable protein

was not inhibited by either 1-deoxymannojirimycin or monensin (data not shown). Collectively, the data indicated that interference with terminal glycosylation did not affect the synthesis or intracellular–extracellular distribution of α-L-fucosidase.

The acidotropic amines, chloroquine, and ammonium chloride interfere with the routing of newly made acid hydrolases in fibroblasts, hepatocytes, macrophages, and monocytes by causing a massive secretion and an intracellular deficiency of these enzymes[4,5,8,9,14,16] (see Scheme 1). The effects of chloroquine and ammonium chloride on trafficking of acid hydrolases in lymphoid cells has not been investigated. Therefore, it was of interest to examine the effects of these compounds on trafficking of α-L-fucosidase in lymphoid cells. In pulse-chase experiments, neither chloroquine nor ammonium chloride interfered with the structural processing of α-L-fucosidase from a precursor form with M_r 58 000 to mature forms having M_r 60 000 and 62 000 (Figs. 2, 4). The compounds did not affect the reactivity of precursor and mature forms of α-L-fucosidase with endo-H (Fig. 3D) (ammonium chloride data not shown). In three pulse-chase experiments, the average percentages of total (intracellular plus extracellular) α-L-fucosidase found extracellularly for cultures treated with 100μM chloroquine and for untreated cultures were 34 and 24%, respectively. In an analogous experiment (Fig. 4), the percentages of total α-L-fucosidase found extracellularly for a culture treated with 2mM ammonium chloride and for an untreated control culture were 10 and 24%, respectively. Thus, neither chloroquine nor ammonium chloride caused massive secretion of α-L-fucosidase relative to control cultures, although chloroquine slightly increased secretion and ammonium chloride slightly decreased secretion. Also, neither 100μM chloroquine nor 2mM ammonium inhibited synthesis of α-L-fucosidase or total protein as judged by incorporation of [^{35}S]methionine into immunoprecipitable α-L-fucosidase or acid-precipitable protein (data not shown). Greater concentrations of chloroquine and ammonium chloride were toxic to lymphoid cells.

DISCUSSION

N-Methyldeoxynojirimycin, 1-deoxymannojirimycin, and monensin interfered with normal processing of asparagine-linked oligosaccharide chains of α-L-fucosidase in lymphoid cells by blocking conversion of endo-H-sensitive oligosaccharides of newly made precursor enzyme to endo-H-resistant oligosaccharides of mature, intracellular and extracellular forms of enzyme. These compounds did not substantially alter the intracellular–extracellular distribution of newly made α-L-fucosidase. This suggested that the sorting of newly made α-L-fucosidase molecules that are retained intracellularly, from molecules that are eventually secreted, did not require terminal glycosylation or the trimming of D-glucose or (1→2)-α-D-linked mannose residues from carbohydrate chains. It is generally accepted that the subcellular delivery route of secretory glycoproteins and lysosomal acid hydrolases is from their site of synthesis in the rough endoplasmic

reticulum to the Golgi complex to either the cell surface or lysosome (reviewed in refs. 5, 6). The carbohydrate chains of glycoproteins acquire resistance to hydrolysis by endo-H in the trans-Golgi. Therefore, the resistance of carbohydrate chains of mature intracellular and extracellular α-L-fucosidase molecules to hydrolysis by endo-H indicated that their sorting normally occurs in the trans-Golgi. However, in cells treated with *N*-methyldeoxynojirimycin, 1-deoxymannojirimycin, or monensin, α-L-fucosidase apparently traversed the trans-Golgi without conversion to endo-H resistance before sorting.

Chloroquine and ammonium chloride had no substantial effect on the intracellular–extracellular distribution of α-L-fucosidase in lymphoid cells. In fibroblasts, hepatocytes, macrophages, and monocytes, these compounds caused a massive secretion and an intracellular deficiency of acid hydrolases[4,5,8,9,14,16]. The action of these weak bases has been attributed to their ability to neutralize the pH of acidic intracellular organelles. The increase in pH apparently retards dissociation of complexes between D-mannopyranosyl phosphate residues of newly made acid hydrolases and phosphomannosyl-intracellular-transport receptors, thereby reducing the amount of unoccupied receptors that deliver acid hydrolases to lysosomes. The different responses to weak bases in lymphoid cells and the other cell types can be explained either by an inability of these agents to neutralize the pH of intracellular organelles in lymphoid cells or by a transport mechanism in lymphoid cells that is independent of pH. The latter would suggest that routing of α-L-fucosidase to lysosomes in lymphoid cells can happen by a mannosyl phosphate-independent pathway. This is consistent with two observations. First, the carbohydrate moiety of the majority of mature, intracellular α-L-fucosidase molecules lacks structures known to harbor the phosphomannosyl-recognition marker[2]. Second, lymphoid cells from I-cell disease patients had normal intracellular levels of acid hydrolases, including α-L-fucosidase, despite an inherited deficiency of an enzyme required for synthesis of the phosphomannosyl-recognition marker[7]. However, since the carbohydrate component of a minority of mature, intracellular α-L-fucosidase molecules contains structures that can harbor the phosphomannosyl-recognition marker, it is possible that routing of α-L-fucosidase in lymphoid cells can occur by both phosphomannosyl-dependent and -independent pathways. The role, if any, of the phosphomannosyl-recognition system in routing α-L-fucosidase in lymphoid cells remains to be determined.

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