The 46-kDa mannose 6-phosphate receptor does not depend on endosomal acidification for delivery of hydrolases to lysosomes

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Summary

In mammalian cells, the mannose 6-phosphate receptor pathway accounts for the transport of most soluble acid hydrolases to lysosomes. It is believed that dissociation of mannose 6-phosphate receptors and their ligands is entirely driven by the acidic environment in endosomal compartments. Indeed, pH-perturbing substances such as ammonium chloride and monensin have been shown to inhibit lysosomal enzyme targeting in cells that express both known mannose 6-phosphate receptors. We now demonstrate that ammonium chloride and monensin exert modest effects on the intracellular retention of lysosomal hydrolases in murine cells that synthesize only the 46-kDa mannose 6-phosphate receptor. Neither ammonium chloride nor monensin induces changes to the subcellular localization of lysosomal hydrolases and the 46-kDa mannose 6-phosphate receptor in these cells. This suggests that endosomal dissociation of the receptor and its ligands still occurs in the presence of these agents. We conclude that the murine 46-kDa mannose 6-phosphate receptor has the capacity to deliver its cargo proteins to lysosomes even in the absence of endosomal acidification.

Key words: Mannose 6-phosphate, Lysosome, Trafficking, Cathepsin, Hydrolase, Biosynthesis

Introduction

Lysosomal delivery of soluble acid hydrolases depends mostly on the presence of mannose 6-phosphate (M6P) in their carbohydrate moieties. These residues mediate binding to specific M6P receptors (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). Two distinct M6P-binding proteins occur in mammalian cells (Kornfeld, 1992), the 300-kDa mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R) and the 46-kDa mannose 6-phosphate receptor (MPR46). Binding of lysosomal enzymes to M6P receptors is thought to occur in the Golgi complex, followed by delivery of the complexes to the lysosomal pathway. Upon reaching endosomes, the ligands dissociate from their receptors which then return to the Golgi stacks. It has been proposed that this segregation process is triggered by the low luminal pH of endosomes because binding of lysosomal enzymes to M6P receptors in vitro is inefficient under acidic conditions (Distler et al., 1991). Treatment of cells with agents that raise the pH in endosomes and lysosomes has been found to reduce lysosomal enzyme sorting (Braulke et al., 1987). Indeed, this has led to the notion that pH-insensitive targeting of lysosomal enzymes is due to an M6P-independent transport pathway (Capony et al., 1994). Evidence for the existence of alternate sorting receptors has been reported (McIntyre and Erickson, 1993). However, isolation and identification of such a protein has remained elusive.

Mice deficient in MPR46 and/or M6P/IGF2R have been generated (Köster et al., 1993; Ludwig et al., 1993; Ludwig et al., 1994). Studies on MPR46- and/or M6P/IGF2R-negative fibroblasts have indicated that both receptors are necessary for efficient lysosomal targeting. It has been suggested that the two receptors complement each other by binding to distinct subpopulations of lysosomal enzymes (Pohlmann et al., 1995). However, sorting by M6P/IGF2R is generally far more efficient than by MPR46, demonstrating that the former is the main lysosomal targeting receptor in mammalian cells. This can be attributed, at least in part, to the lower affinity of MPR46 for multivalent M6P-containing ligands, compared with M6P/IGF2R (Tong and Kornfeld, 1989; Tong et al., 1989).

We have previously reported that in M6P/IGF2R-deficient murine SCC-VII squamous carcinoma cells, lysosomal enzyme trafficking is largely insensitive to the pH-perturbing agents NH_4Cl and chloroquine (Lorenzo et al., 2000). We now provide evidence that in these and other M6P/IGF2R-deficient murine cells, transport of acid hydrolases to lysosomes is dependent on MPR46, and show that this pathway is functional in the absence of endosomal acidification.

Results

Intracellular sorting of cathepsin B is strictly dependent on N-glycosylation of the protein

To investigate the role of *N*-linked oligosaccharides in the biosynthesis of cathepsin B in M6P/IGF2R-deficient cells, SCC-VII cells were metabolically labeled with

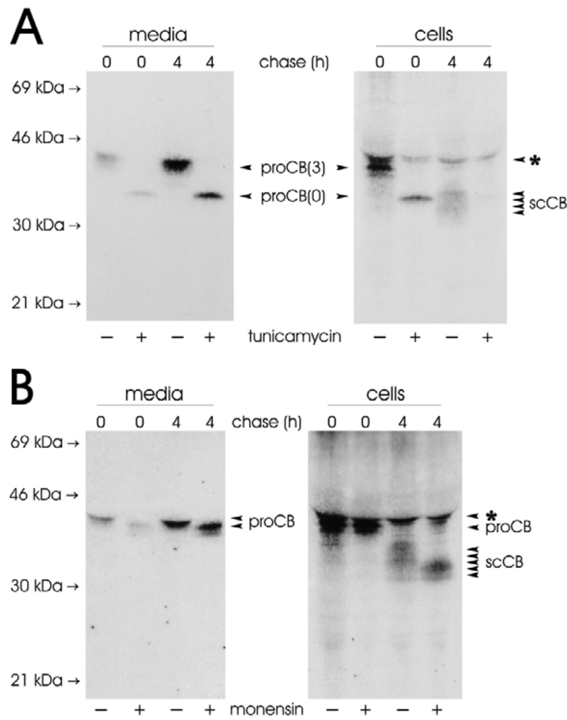

Fig. 1. Effects of tunicamycin and monensin on cathepsin B biosynthesis in SCC-VII cells. Confluent monolayers of SCC-VII cells were metabolically labeled for 1 hour with 100 μCi/ml [^{35}S]methionine and subsequently chased for 4 hours in the absence (–) or continuous presence (+) of 10 μg/ml tunicamycin (A) or 1 μM monensin (B). Cathepsin B was then immunoprecipitated from equivalent amounts of cell and medium extracts and analyzed by SDS-PAGE and fluorography. proCB, procathepsin B; proCB(3), fully glycosylated procathepsin B carrying 3 *N*-linked oligosaccharide side chains; proCB(0), unglycosylated procathepsin B; scCB, mature cathepsin B (single-chain form). Experiments using cathepsin-B-specific murine embryonic fibroblasts (MEFs) revealed that the band labeled with an asterisk represents a polypeptide unrelated to cathepsin B, which is sometimes nonspecifically co-precipitated from cell extracts by the antiserum used in these studies. Note that this polypeptide is not present in cathepsin B immunoprecipitates from culture media. The migration positions of ^{14}C-labeled molecular mass standards are indicated.

[^{35}S]methionine in the absence or presence of tunicamycin, an inhibitor of protein *N*-glycosylation. In the absence of the drug, immunoprecipitation with antibodies against cathepsin B detected a 42-kDa form of the protein in the cell lysate at the start of the chase. This polypeptide corresponds to glycosylated procathepsin B, the latent precursor of the proteinase [Fig. 1A, proCB(3)]. After 4 hours of chase, the fraction retained inside the cells was completely processed to lower molecular mass bands (Fig. 1A, scCB), which represent different glycoforms of the mature proteinase (Lorenzo et al., 2000). However, 60% of the newly synthesized proenzyme was secreted into the medium as a 44-kDa polypeptide.

Upon tunicamycin treatment, procathepsin B was present as a 36-kDa polypeptide in cells and medium before the chase [Fig. 1A, proCB(0)]. This apparent molecular mass is identical to that of enzymatically deglycosylated procathepsin B (Lorenzo et al., 2000) and agrees well with the theoretical molecular mass of the non-glycosylated proenzyme (Chan et al., 1986). After a 4-hour chase, the tunicamycin-treated cells had secreted virtually all (>97%) labeled procathepsin B, with no mature enzyme detectable within the cells even after long exposure times (Fig. 1A). Since procathepsin B delivered to the lysosomal pathway is rapidly converted into the mature forms of the proteinase (Schmid et al., 1999), these results indicate that in SCC-VII cells, biosynthetic transport of cathepsin B to lysosomes strictly relies on the presence of a receptor binding to the carbohydrate moiety of the enzyme.

Lysosomotropic amines and monensin exert only weak effects on lysosomal enzyme sorting in M6P/IGF2R-deficient cell lines

We have previously found that in SCC-VII cells lysosomal trafficking of cathepsin B and other acid hydrolases is largely insensitive to the pH-perturbing agents NH_4Cl and chloroquine (Lorenzo et al., 2000). The carboxylic ionophore monensin is also known to dissipate the transmembrane pH gradients in the Golgi and lysosomal compartments (Braulke et al., 1987). Secretion of newly synthesized procathepsin B by SCC-VII cells upon monensin treatment (40%) was only slightly higher than by control cultures (31% in this experiment). Thus, monensin cannot further impede the residual intracellular retention of lysosomal enzymes in these

Table 1. Effect of NH_4Cl on cathepsin B secretion

Cell line	IGF2R status	MPR46 status	CathB secretion (%) control	CathB secretion (%) + NH_4Cl	NH_4Cl-induced increase in CathB secretion (%)
SCC-VII	–	+	58±9 (5)	61±7 (5)	3±7
MEF	–	+	55 (1)	70 (1)	15
RAW 264.7	<5%	+	58 (1)	62 (1)	4
3T3-L1	+	+	8±2 (2)	40±2 (2)	32±4
NIH/3T3	+	+	1 (1)	52 (1)	51
L-M(TK$^-$)	+	+	13 (1)	43 (1)	30
Mean (IGF2R$^-$)	–	+	57±1	64±3	7±4*
Mean (IGF2R$^+$)	+	+	7±3	45±4	38±7*
MEF	–	–	>90	n.a.	n.a.

Cells were metabolically labeled with [^{35}S]methionine for 1 hour and subsequently chased for 5 hours in the continuous absence (control) or presence of 10 mM NH_4Cl. Cathepsin B was then immunoprecipitated from cell and medium extracts and analyzed by SDS-PAGE and fluorography. Band intensities were determined by densitometric analysis of the respective films. The relative M6P/IGF2R content of RAW 264.7 cells was estimated by immunoblotting in comparison to L-M(TK$^-$), NIH/3T3 and 3T3-L1 fibroblasts. Data are presented as mean ± s.e.m., with the number of experiments listed in parentheses. Statistical analysis was performed using Student's *t*-test. CathB, cathepsin B; IGF2R, M6P/IGF2R; *P<0.02; n.a., not analyzed.

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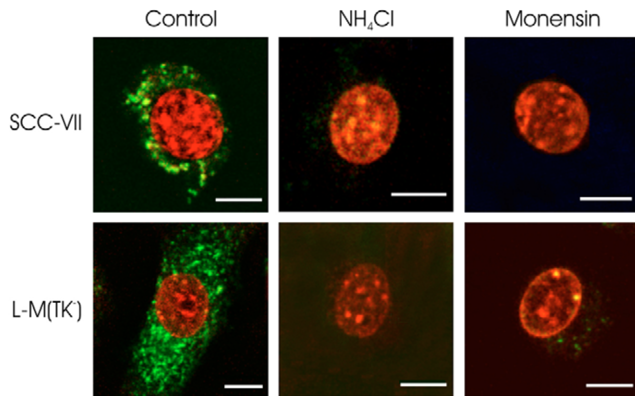

Fig. 2. Effects of NH_4Cl and monensin on acidic organelles in SCC-VII cells and L-M(TK$^-$) fibroblasts. SCC-VII cells and M6P/IGF2R-positive L-M(TK$^-$) fibroblasts were incubated in complete culture medium in the absence (control) or presence of either 10 mM NH_4Cl or 1 μM monensin for 2 hours at 37°C. The cells were then labeled with LysoSensor Yellow/Blue (which accumulates in acidic compartments; pseudocolored in green) and DAPI (which stains nuclei; pseudocolored in red) before analysis by confocal laser-scanning microscopy. Bars, 10 μm.

M6P/IGF2R-deficient cells (Fig. 1B). These results mimic the previously reported effects of NH_4Cl and chloroquine on biosynthetic cathepsin B transport in SCC-VII cells (Lorenzo et al., 2000) (Table 1). However, treatment with NH_4Cl or monensin abolished the staining of SCC-VII lysosomes with Lysosensor Yellow/Blue, a dye which specifically accumulates in acidic organelles. The same results were obtained for M6P/IGF2R-positive L-M(TK$^-$) fibroblasts. This indicates that NH_4Cl and monensin are effective in elevating the lysosomal pH in SCC-VII cells to a similar extent as in M6P/IGF2R-positive cells (Fig. 2).

The above results prompted us to study the effect of NH_4Cl on the intracellular transport of newly synthesized lysosomal enzymes in other murine cell lines either lacking M6P/IGF2R or expressing merely very low levels of the receptor. When the biosynthesis of cathepsin B in *Igf2r*$^{-/-}$ murine embryonic fibroblasts (MEFs) was investigated, substantial amounts (55%) of newly synthesized procathepsin B were detected in the culture medium (Fig. 3A). Secretion of the cathepsin B precursor was only modestly stimulated by NH_4Cl (70%). Similar results were obtained for M6P/IGF2R-deficient RAW 264.7 macrophages. By contrast, the low level of procathepsin B secretion by M6P/IGF2R-positive cells was distinctly elevated in the presence of NH_4Cl (Table 1).

We also investigated the effect of NH_4Cl on the secretion and processing of other lysosomal proteinases in M6P/IGF2R-positive NIH/3T3 fibroblasts and *Igf2r*$^{-/-}$ MEFs. NIH/3T3 cells secreted only limited amounts of procathepsin D (<1%) and procathepsin L (5%), which were distinctively enhanced by the addition of NH_4Cl (17% and 75%, respectively; not shown). By contrast, *Igf2r*$^{-/-}$ MEFs deliver large fractions of their newly synthesized procathepsin D (50%) and L (61%) into the culture medium. Secretion of procathepsin D and L by these cells was only slightly stimulated by the presence of NH_4Cl (57% and 67%, respectively). However, proteolytic maturation of intracellular procathepsin L was dramatically impaired by the addition of the base (Fig. 3B), indicating that NH_4Cl is effective in elevating the lysosomal pH in *Igf2r*$^{-/-}$ MEFs.

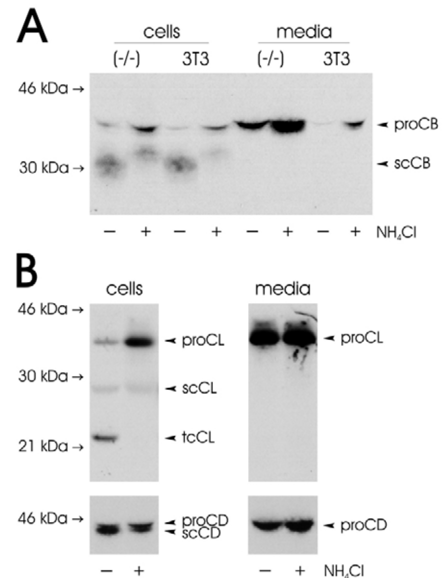

Fig. 3. Effect of NH_4Cl on the biosynthesis of cathepsin B, D and L in NIH/3T3 cells and M6P/IGF2R-negative mouse embryonic fibroblasts. (A) *Igf2r*$^{-/-}$ MEFs and receptor-positive NIH/3T3 cells were metabolically labeled with [^{35}S]methionine and chased for 5 hours in the absence (–) or continuous presence (+) of 10 mM NH_4Cl as described in Fig. 1. Cathepsin B was then immunoprecipitated from equivalent amounts of cell and medium extracts and analyzed by SDS-PAGE followed by fluorography. Note that the nonspecific band described in Fig. 1 was not observed in this experiment. proCB, procathepsin B; scCB, mature cathepsin B (single-chain form). (B) *Igf2r*$^{-/-}$ MEFs were metabolically labeled with [^{35}S]methionine and chased for 5 hours in the absence (–) or continuous presence (+) of 10 mM NH_4Cl as described in Fig. 1. Cathepsin D and L were then sequentially immunoprecipitated from equivalent amounts of cell and medium extracts and analyzed by SDS-PAGE followed by fluorography. proCL, procathepsin L; scCL, mature cathepsin L (single-chain form); tcCL, heavy-chain of mature cathepsin L (two-chain form); proCD, procathepsin D; scCD, mature cathepsin D (single-chain form).

In line with the results obtained for cathepsin B, D and L, treatment of various M6P/IGF2R-deficient cell lines with NH_4Cl led at best to a slightly stimulated release of the classical lysosomal marker enzyme, β-*N*-acetylhexosaminidase. In fact, even the NH_4Cl-treated cultures secreted a substantially smaller fraction of their β-*N*-acetylhexosaminidase activity than MEFs lacking both M6P receptors (Table 2). These data support the hypothesis that pH-perturbing agents such as lysosomotropic amines and monensin generally interfere only weakly with the residual M6P-mediated lysosomal targeting of acid hydrolases in M6P/IGF2R-deficient cell lines.

Biosynthetic delivery of cathepsin B, D and L to lysosomes in murine fibroblasts is strictly dependent on M6P receptors

The biosynthesis of cathepsin B, D and L was also studied in MEFs lacking both known M6P receptors, M6P/IGF2R and MPR46. In contrast to M6P/IGF2R-deficient MEFs (which contain normal amounts of MPR46), M6P receptor (MPR)-

Table 2. Effect of NH_4Cl on β-*N*-acetylhexosaminidase secretion

Cell line	IGF2R status	MPR46 status	Hex secretion (%) control	Hex secretion (%) + NH_4Cl	NH_4Cl-induced increase in Hex secretion (%)
SCC-VII	–	+	59±6 (4)	70±3 (4)	11±4
MEF	–	+	49±1 (2)	60±2 (2)	11±1
L (D9)	–	+	15±2 (4)	17±6 (4)	2±5
P388D$_1$	–	+	43±12 (2)	35±8 (2)	0
RAW 264.7	<5%	+	28±3 (4)	35±3 (4)	7±3
SCC/IGF2R	+	+	14±3 (4)	57±5 (4)	43±5
3T3-L1	+	+	3±1 (3)	30±3 (3)	27±3
NIH/3T3	+	+	3±1 (4)	36±6 (4)	33±5
L-M(TK$^-$)	+	+	10±2 (4)	21±3 (4)	11±2
Mean (IGF2R$^-$)	–	+	39±8	43±10	6±2*
Mean (IGF2R$^+$)	+	+	8±3	36±8	28±7*
MEF	–	–	92±2 (4)	91±1 (4)	0

Cells were incubated for 24 hours in complete culture medium in the absence (control) or presence of 10 mM NH_4Cl. The activity of β-*N*-acetylhexosaminidase was then determined in the respective cell homogenates and conditioned culture media. The relative M6P/IGF2R content of RAW 264.7 cells was estimated by immunoblotting in comparison to L-M(TK$^-$), NIH/3T3 and 3T3-L1 fibroblasts. Data are presented as mean ± s.e.m., with the number of experiments provided in parentheses. Statistical analysis was performed using Student's *t*-test. SCC/IGF2R, SCC-VII cells stably transfected with human M6P/IGF2R cDNA; Hex, β-*N*-acetylhexosaminidase; IGF2R, M6P/IGF2R; *P<0.02.

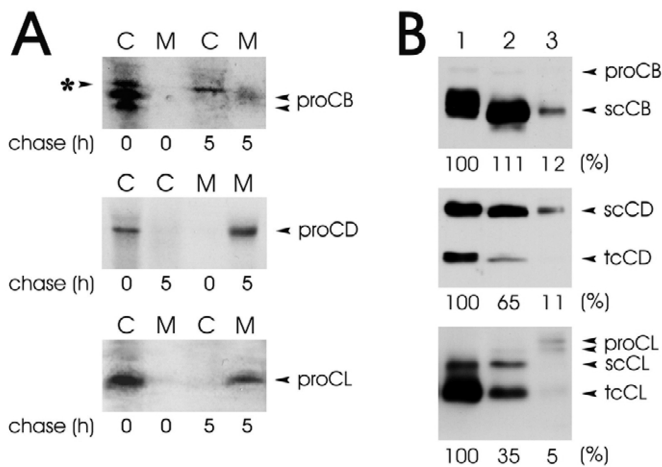

Fig. 4. Biosynthesis of cathepsin B, D and L in MPR-negative mouse embryonic fibroblasts. (A) MPR-negative MEFs lacking both M6/IGF2R and MPR46 were metabolically labeled with [^{35}S]methionine for 1 hour and then chased for up to 5 hours as described in Fig. 1. Procathepsin B (40-42 kDa), procathepsin D (46 kDa) and procathepsin L (37 kDa) were then sequentially immunoprecipitated from equivalent amounts of cell and medium extracts and analyzed by SDS-PAGE and fluorography. The mature forms of the proteinases were not detected in this experiment. The band labeled with an asterisk is nonspecific as described in Fig. 1. proCB, procathepsin B; proCD, procathepsin D; proCL, procathepsin L. (B) Microsomal extracts (15 μg total protein) of L-M(TK$^-$) cells (lane 1), *Igf2r*$^{-/-}$ MEFs (lane 2) and MPR-negative MEFs (lane 3) were separated by SDS-PAGE and analyzed by immunoblotting with antibodies to cathepsin B, cathepsin D and cathepsin L. Band intensities determined by densitometry were used to compare the antigen contents of the samples. proCB, procathepsin B (42 kDa); scCB, mature cathepsin B (single-chain form; 30-32 kDa); scCD, mature cathepsin D (single-chain form; 43 kDa); tcCD, heavy-chain of mature cathepsin D (two-chain form; 29 kDa); proCL, procathepsin L (34-36 kDa); scCL, mature cathepsin L (single-chain form; 29 kDa); tcCL, heavy-chain of mature cathepsin L (two-chain form; 21 kDa).

negative MEFs secrete more than 90% of their newly synthesized procathepsin B. The same observations were made for procathepsin D and L (Fig. 4A; Table 1). MPR-negative MEFs also secrete more than 90% of their β-*N*-acetylhexosaminidase activity (Table 2). For cathepsin D and β-*N*-acetylhexosaminidase, similar results have been reported previously (Pohlmann et al., 1995; Dittmer et al., 1999). These findings are in good agreement with the drastically reduced specific activity of β-*N*-acetylhexosaminidase (10%) in MPR-negative MEFs compared with normal murine fibroblasts (data not shown) (Pohlmann et al., 1995; Dittmer et al., 1999). Similar results were obtained for cathepsin B, D and L by immunoblot analysis, which also revealed that proteolytic maturation of cathepsin L and to a lesser extent cathepsin D is partially impaired in MPR-negative fibroblasts (Fig. 4B).

These results establish that biosynthetic transport of cathepsin B and L in murine fibroblasts relies entirely on the M6P receptor system, as previously observed for cathepsin D and β-*N*-acetylhexosaminidase (Pohlmann et al., 1995; Dittmer et al., 1999). This implies that in general the residual intracellular retention of lysosomal enzymes in M6P/IGF2R-deficient MEFs is mediated by the second M6P receptor, MPR46.

Treatment with lysosomotropic amines and monensin does not prevent the formation of the M6P recognition marker

The synthesis of the M6P recognition marker is initiated by the cis-Golgi enzyme UDP-*N*-acetylglucosamine-1-phosphotransferase (von Figura and Hasilik, 1986; Tiede et al., 2005). However, generation of exposed M6P residues requires the subsequent action of the 'uncovering enzyme' *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase – a protein that resides in the trans-Golgi network (TGN) (Rohrer and Kornfeld, 2001). We have previously shown that SCC-VII cells are capable of forming functional M6P residues on lysosomal enzymes (Lorenzo et al., 2000). To assess the effects of NH_4Cl and monensin on the phosphorylation status of lysosomal enzymes, SCC-VII cells

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Table 3. Effect of NH_4Cl and monensin on the phosphorylation status of secreted SCC-VII glycoproteins

N-glycan fraction (Endo-H-sensitive)	Control (%)	NH_4Cl (%)	Monensin (%)
Uncharged	35	37	40
1 Sia	2	1	n.d.
1 PD	6	14	21
1 PD, 1 Sia	1	3	n.d.
2 PD	n.d.	3	14
1 PM	13	8	7
1 PM, 1 PD	2	6	9
1 PM, 1 Sia	3	n.d.	n.d.
2 PM	38	28	9
Total (phosphorylated)	63	62	60
Uncovering efficiency	92	71	37

SCC-VII cells were metabolically labeled with [^{3}H]mannose in the absence (control) or presence of either 10 mM NH_4Cl or 1 μM monensin. Oligomannosidic and hybrid *N*-glycans were released from secreted glycoproteins by treatment with endo-β-*N*-acetylglucosaminidase H (Endo H) and fractionated by anion-exchange chromatography. Individual fractions were characterized by mild acid treatment (to convert phosphodiesters into phosphomonoesters) as well as digestion with sialidase and alkaline phosphatase. The uncovering efficiency was calculated as the fraction of phosphate groups being phosphomonoesters. Sia, sialic acid; PD, phosphodiester; PM, phosphomonoester; n.d., not detectable (<0.5%).

were metabolically labeled with [^{3}H]mannose in the absence (control) or presence of the drugs. ^{3}H-labeled glycoproteins secreted into the culture medium were then isolated and treated with endo-β-*N*-acetylglucosaminidase H (Endo H) to release high-mannose and hybrid-type *N*-linked oligosaccharides. By means of anion-exchange chromatography, the control sample was found to consist of neutral (35%), sialylated (2%) and phosphorylated (63%) *N*-glycans (Table 3). Of the carbohydrate-associated phosphate groups synthesized by untreated SCC-VII cells, 92% were found to be phosphomonoesters which serve as high-affinity ligands for M6P receptors (Kornfeld and Mellman, 1989). Treatment with NH_4Cl did not affect the sialylation and phosphorylation of Endo-H-sensitive *N*-linked oligosaccharides. However, the ‘uncovering’ efficiency of phosphorylated *N*-glycans was moderately reduced (71%). This is in good agreement with data reported for human and murine fibroblasts (Isidoro et al., 1990; Dittmer and von Figura, 1999). Monensin displayed a more pronounced effect on the composition of Endo-H-sensitive *N*-glycans, because sialylated structures were not detected upon treatment with the drug. This is in accordance with previous findings (Dittmer and von Figura, 1999). By contrast, phosphorylation of *N*-glycans was not significantly affected by monensin treatment. However, monensin substantially reduced the ‘uncovering’ of M6P residues, with only 37% of them present as phosphomonoester groups (Table 3). Similar observations have been made previously with murine fibroblasts (Dittmer and von Figura, 1999). These results demonstrate that NH_4Cl and monensin do not impede the activity of UDP-*N*-acetylglucosamine-1-phosphotransferase, but inhibit the action of the ‘uncovering enzyme’, *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase. In fact, the moderate reduction of the ‘uncovering’ efficiency by NH_4Cl could account for the slight stimulatory effect of this compound on the secretion of cathepsin B (Table 1) and β-*N*-acetylhexosaminidase (Table 2) by SCC-VII cells and other M6P/IGF2R-deficient cell lines.

Lysosomotropic amines and monensin do not change the subcellular localization of lysosomal enzymes and their receptors in SCC-VII cells

To assess the subcellular distribution of lysosomal enzymes in SCC-VII cells treated with NH_4Cl and monensin, post-nuclear organelles were separated by Percoll density-gradient centrifugation. In all gradients, the activity of the lysosomal marker β-*N*-acetylhexosaminidase was evenly distributed between heavy (lysosomal) and light (endosomal) fractions (Fig. 5). By contrast, the bulk of the activities of the Golgi enzyme galactosyltransferase and the ER marker NADH-cytochrome-*c*-reductase were located in the light gradient fractions (not shown). Immunoblot analysis revealed the presence of cathepsin D in heavy and light gradient fractions. Treatment of SCC-VII cells with monensin induced a subtle reduction of the amount of cathepsin D residing in lysosomes. This effect was not observed upon NH_4Cl treatment (Fig. 5). Similar results were observed for the effects of NH_4Cl and monensin on the subcellular distribution of cathepsin L (not shown).

In addition to endosomes, the light Percoll gradient fractions contain a range of other cellular compartments such as the Golgi complex. Hence, it could be envisioned that

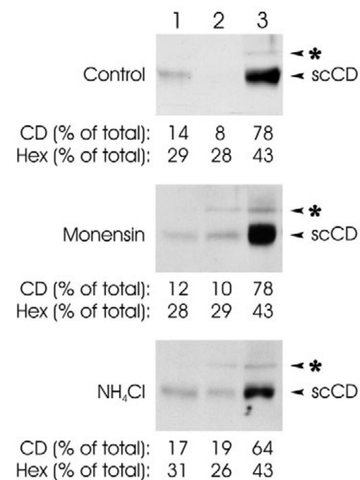

Fig. 5. Effects of NH_4Cl and monensin on the endosomal/lysosomal distribution of cathepsin D in SCC-VII cells. SCC-VII cells were incubated in complete culture medium in the absence (control) or presence of either 10 mM NH_4Cl or 1 μM monensin for 10 hours at 37°C. Post-nuclear supernatants were obtained and fractionated by density-gradient centrifugation. Pooled heavy (lane 1), intermediate (lane 2) and light (lane 3) gradient fractions were then tested for β-*N*-acetylhexosaminidase (Hex) activity and subjected to immunoblotting with antibodies to cathepsin D (CD). Band intensities were determined by densitometric analysis of the respective films. Data are presented as the mean values of two to four experiments. One representative blot is shown in each case. The band labeled with an asterisk represents a polypeptide unrelated to cathepsin D that was nonspecifically detected in these experiments. Markers for the Golgi apparatus and the endoplasmic reticulum were largely confined to the light gradient fractions. scCD, mature cathepsin D (single-chain form; 43 kDa).

treatment with NH_4Cl or monensin causes a partial relocation of endosomal cathepsin D to the TGN. However, this possibility was ruled out by indirect immunofluorescence analysis of SCC-VII cells treated with these compounds. As in control cells, immunocytochemical detection of cathepsin D revealed numerous vesicles distributed throughout the cytoplasm, a staining pattern reminiscent of endosomes and lysosomes. This was verified by the extensive colocalization of cathepsin D and the endosomal/lysosomal marker LAMP-1 (Fig. 6). By contrast, the TGN marker TGN38 was found concentrated in the perinuclear region, as typically observed for Golgi-derived elements. Importantly, significant overlap of the subcellular distributions of cathepsin D and TGN38 was not detected either in controls or in NH_4Cl- or monensin-treated cells (Fig. 7A). These results demonstrate that treatment with either NH_4Cl or monensin does not result in a significant relocation of soluble endosomal and lysosomal hydrolases to the TGN and other regions of the Golgi apparatus.

It has been reported that in acidification-incompetent human cancer cells, M6P/IGF2R is mainly found in endosomes and lysosomes. Similarly, NH_4Cl treatment of acidification-competent human cancer cells led to a redistribution of Golgi-associated M6P/IGF2R to endosomal and lysosomal structures (Kokkonen et al., 2004). When untreated SCC-VII cells were double-stained with antibodies to MPR46 and LAMP-1, no co-localization of the two antigens was observed. Furthermore, neither NH_4Cl nor monensin led to significant colocalization of MPR46 and LAMP-1 in SCC-VII cells (Fig. 7B). As in untreated cells, MPR46 was found concentrated in perinuclear Golgi-like structures and extensively colocalized with the TGN marker TGN38 (data not shown). These findings indicate that endosomal dissociation of MPR46 and its ligands is not prevented by NH_4Cl and monensin.

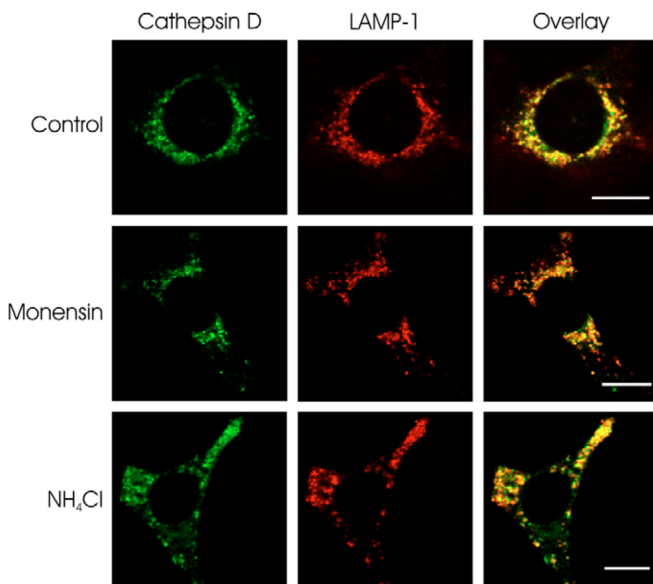

Fig. 6. Effects of NH_4Cl and monensin on the subcellular localization of cathepsin D and LAMP-1 in SCC-VII cells. SCC-VII cells were incubated in complete culture medium in the absence (control) or presence of either 1 μM monensin or 10 mM NH_4Cl for 10 hours at 37°C. The cells were then fixed, permeabilized and incubated with antibodies to cathepsin D and LAMP-1. Bound primary antibodies were then detected with FITC- and Cy3-labeled secondary antibodies before analysis of the immunostained cells by confocal laser-scanning microscopy. Bars, 10 μm.

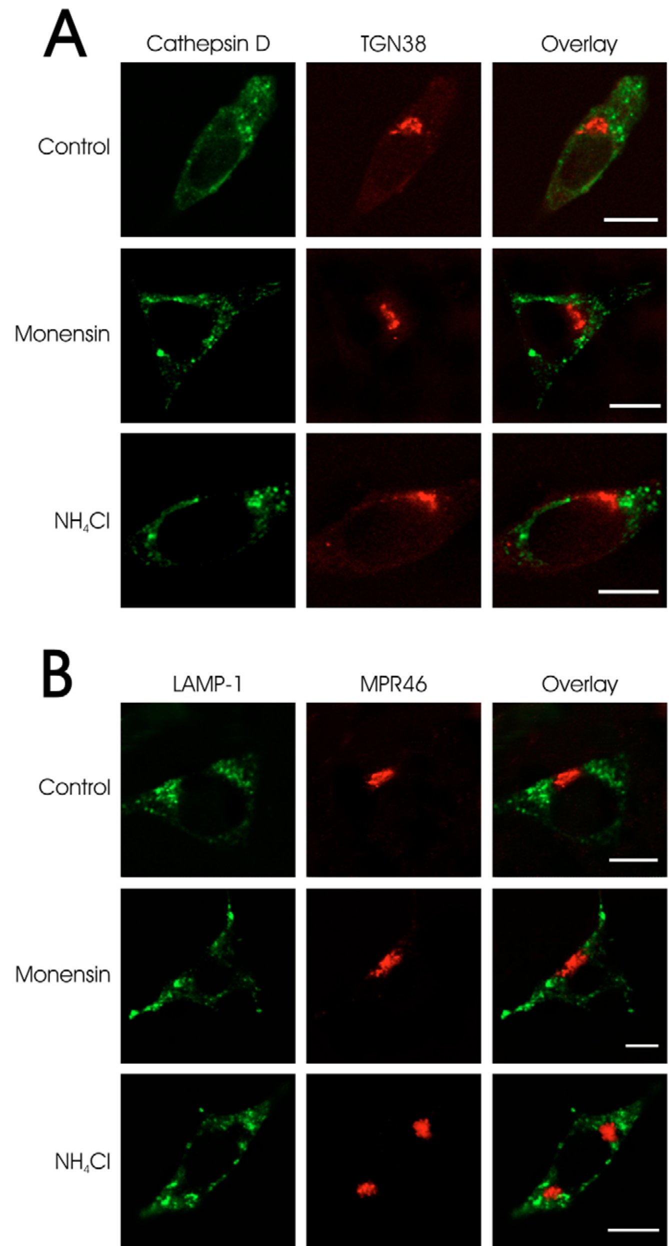

Fig. 7. Effects of NH_4Cl and monensin on the subcellular localization of MPR46 and its ligands in SCC-VII cells. SCC-VII cells were incubated in complete culture medium in the absence (control) or presence of either 1 μM monensin or 10 mM NH_4Cl for 10 hours at 37°C. The cells were then fixed, permeabilized and incubated with antibodies to cathepsin D and TGN38 (panel A), or antibodies to MPR46 and the lysosomal marker LAMP-1 (panel B). Bound primary antibodies were then detected with FITC- and Cy3-labeled secondary antibodies before analysis of the immunostained cells by confocal laser-scanning microscopy. Bars, 10 μm.

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Discussion

We demonstrate in this report that the intracellular targeting of cathepsin B and other lysosomal proteinases in M6P/IGF2R-negative murine fibroblasts and SCC-VII cells relies on the presence of MPR46. Similar results have been reported for M6P/IGF2R-deficient rat Morris 7777 hepatoma cells, where treatment with blocking antibodies against MPR46 induced the secretion of the majority of the residual intracellular acid hydrolases (Stein et al., 1987). This supports the concept that at least in the cell types investigated, biosynthetic transport of acid hydrolases to lysosomes strictly requires the presence of the M6P recognition marker and the expression of at least one of the two M6P receptors.

M6P-dependent sorting of lysosomal hydrolases is thought to be initiated in the TGN. In this compartment, M6P-containing ligands first associate with M6P receptors before packaging into clathrin-coated vesicles for transport to late endosomes where the receptor-ligand complexes dissociate (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). This process might be triggered by differences in the luminal pH of these compartments. The TGN displays a pH of 6.0-6.5, whereas late endosomes have a pH of 5.0-5.5 (Mellman et al., 1986; van Weert et al., 1995; Miesenböck et al., 1998). Binding of ligands to immobilized M6P receptors is strongly reduced at pH 5.4 compared with the optimal pH range for this interaction (pH 6.2-6.6) (Distler et al., 1991; Pohlmann et al., 1995). Hence, carboxylic ionophores and lysosomotropic amines that increase the luminal pH of lysosomes to pH 6.0-6.3 (Yoshimori et al., 1991; Moriyama et al., 1992) should prevent the displacement of the enzymes from their receptors and thus impede lysosomal hydrolase sorting. We now show that such compounds fail to prevent the MPR46-dependent residual lysosomal targeting of M6P-modified proteinases in M6P/IGF2R-deficient cells. This suggests that the pH-gradient in the endosomal/lysosomal system is not solely responsible for dissociation of MPR46 and its ligands in situ. Other factors, such as the structural properties of the receptor or intrinsic features of the endosomal/lysosomal system, are probably also involved in this process.

One such factor might be the oligomerization status of MPR46 as oligomeric forms of the receptor seem to display a higher affinity to M6P-containing ligands than dimeric MPR46. Although the receptor exists mostly as a homodimer, substantial amounts of tetramers can be detected in purified MPR46 preparations (Waheed and von Figura, 1990). In vitro, tetramer formation is favored, amongst other factors, by a high receptor concentration and the presence of Mn^{2+} ions (Waheed et al., 1990; Sun et al., 2005). Because MPR46 is mainly localized in the TGN (Klumperman et al., 1993) and thus occurs there at a higher local concentration than in endosomal compartments, it could be that TGN-derived MPR46 tetramers spontaneously dissociate upon arrival in endosomes and thus release bound ligands. A similar scenario could be envisaged for Mn^{2+}-driven differences between the quaternary structures of MPR46 in the TGN and endosomes. However, no major changes in the tetramer/dimer ratio of MPR46 could be detected during intracellular recycling and ligand binding (Punnonen et al., 1996).

Another potential means to trigger pH-insensitive receptor-ligand dissociation could be based on the intrinsic ligand-binding properties of MPR46. MPR46 binds multivalent M6P-containing ligands less avidly than M6P/IGF2R (Tong and Kornfeld, 1989; Tong et al., 1989; Distler et al., 1991). Thus, the secretion of substantial amounts of lysosomal hydrolases by M6P/IGF2R-deficient cells could be due to low-affinity interactions of the enzymes with MPR46. Equilibrium dialysis experiments have revealed that MPR46 binds to bovine testis β-galactosidase and a high-mannose oligosaccharide carrying two phosphomonoesters only with a K_d of 2×10^{-7} M (Tong and Kornfeld, 1989; Distler et al., 1991). Lysosomal hydrolases carrying one or two phosphorylated oligomannosidic *N*-glycans such as procathepsin B (Lorenzo et al., 2000) should display a similar affinity to MPR46. The concentration of procathepsin B in the TGN can be estimated to reach 1×10^{-6} M, assuming a cellular synthesis rate of 1×10^{-19} mol procathepsin B per hour (Coulibaly et al., 1999), a cellular volume of 1×10^{-12} liter of which 10% is accounted for by the compartments involved in secretion (Griffiths et al., 1989), and a transition time of 1 hour in the secretory pathway (Hanewinkel et al., 1987). This concentration would be sufficient to permit binding to MPR46 and thus enforce segregation to the lysosomal pathway. Once delivered to endosomes, ligand displacement could be initiated by dilution of the complexes, thus enforcing enhanced ligand dissociation. Subsequent dephosphorylation because of the action of endosomal phosphatases would prevent reassociation of ligands and MPR46. It has been shown that M6P-modified acid hydrolases can be quickly dephosphorylated upon delivery to late endosomes (Einstein and Gabel, 1991). Thus, it is possible that at least three factors contribute to MPR46-ligand dissociation in the absence of a pH-gradient: the oligomerization status of the receptor, differences in the concentration of M6P-modified lysosomal hydrolases in situ, and the presence of phosphatases in the recipient compartments.

Materials and Methods

Reagents

Tran[^{35}S]label metabolic labeling reagent (>1000 Ci/mmol) was obtained from ICN Pharmaceuticals (Costa Mesa, CA). [^{3}H]mannose (10-20 Ci/mmol), UDP-[^{3}H]galactose (5-20 Ci/mmol), ^{14}C-methylated molecular mass standards, Amplify™ fluorographic reagent, Percoll and protein-A-Sepharose 4B were provided by Amersham Biosciences (Rainham, UK). Hygromycin B, NADH (disodium salt) and tunicamycin were from Roche Diagnostics (Mannheim, Germany). Prestained molecular mass standards and enhanced chemiluminescence western blotting reagents were obtained from Bio-Rad (Richmond, CA). E-64, leupeptin, 4-methylumbelliferyl-β-*N*-acetylglucosaminide, monensin, phenylmethylsulfonyl fluoride (PMSF) and sodium β-glycerophosphate were purchased from Sigma (St Louis, MO). All other chemicals were of reagent grade.

Antibodies

A rabbit antiserum against human cathepsin B (Mach et al., 1992) has been shown to crossreact with all forms of the murine enzyme (Coulibaly et al., 1999). The other rabbit antisera used in this study were: anti-mouse cathepsin D [(Pohlmann et al., 1995) or John S. Mort, Shriners Hospital for Children, Montreal, Canada]; anti-mouse procathepsin L (Ann H. Erickson, University of North Carolina, Durham, NC); anti-bovine M6P/IGF2R (Bernard Hoflack, Technische Universität Dresden, Germany); anti-mouse MPR46 (Annette Hille-Rehfeld, Georg-August Universität Göttingen, Germany). The rat monoclonal antibody to mouse LAMP-1 (clone 1D4B) developed by J. Thomas August (Johns Hopkins University, Baltimore, MD) was obtained as culture supernatant from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences (University of Iowa, Iowa City, IA). Affinity-purified sheep antibodies to rat TGN38 were from Serotec (Oxford, UK).

Cell culture

Murine SCC-VII squamous carcinoma cells (Coulibaly et al., 1999) were propagated in Minimal Essential Medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The

following cell lines were maintained in Dulbecco's Modified Eagle's medium with the same supplements: murine RAW 264.7 macrophages and L-M(TK$^-$) fibroblasts (American Type Culture Collection, Manassas, VA); M6P/IGF2R-deficient murine L cells (clone D9; Jack Rohrer, Universität Zürich, Switzerland); immortalized *Igf2r*$^{-/-}$ mouse embryonic fibroblasts (Erwin Wagner, Research Institute of Molecular Pathology, Vienna, Austria); Simian virus 40 (SV40)-transformed mouse embryonic fibroblasts lacking M6P/IGF2R and MPR46 (Kasper et al., 1996); SV40-transformed mouse embryonic fibroblasts lacking cathepsin B, D or L (Christoph Peters, Albert-Ludwigs-Universität, Freiburg, Germany). Murine NIH/3T3 (American Type Culture Collection) and 3T3-L1 fibroblasts (David James, University of Queensland, Brisbane, Australia) were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% bovine calf serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Murine P388D$_1$ macrophages (American Type Culture Collection) were grown in RPMI 1640 medium containing 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All tissue culture reagents were purchased from Invitrogen (Carlsbad, CA).

Generation of SCC-VII cells stably transfected with human M6P/IGF2R cDNA

All molecular biology techniques were performed following standard procedures. A cDNA encoding the complete human M6P/IGF2R open reading frame (American Type Culture Collection) was subcloned into the plasmid pCDM8 (Invitrogen). This construct (obtained from Vladimir Leksa and Hannes Stockinger, Institute of Immunology, Medical University of Vienna, Austria) was digested with the restriction enzyme *Xba*I (Fermentas, St Leon-Rot, Germany) to excise its 8-kb insert. T4 DNA ligase (Fermentas) was used to ligate this fragment into the *Xba*I site within the multiple cloning region of pAHygCMV2 (Coulibaly et al., 1999). SCC-VII cells were transfected with the resulting vector using Lipofectin (Invitrogen) according to the manufacturer's instructions. Selection of stably transfected cells was achieved by virtue of their ability to grow in the presence of 350 μg/ml hygromycin B. Drug-resistant clones were isolated after 14 days and tested for M6P/IGF2R production by immunofluorescence analysis. One of the positive clones (clone 15) was selected for further experiments.

Metabolic labeling and immunoprecipitation

Confluent cell monolayers were metabolically labeled for 1 hour with [^{35}S]methionine (100 μCi/ml), then chased for 4-5 hours as described (Hanewinkel et al., 1987; Lorenzo et al., 2000). When added, the following compounds were present throughout the entire experimental procedure: NH_4Cl (10 mM), monensin (1 μM) and tunicamycin (10 μg/ml). In the case of monensin and tunicamycin, the control cultures were treated with the solvent (dimethyl sulfoxide) at a final concentration of 1% (v/v).

Immunoprecipitation of antigens from equivalent amounts of labeled cell and medium extracts followed by SDS-PAGE and fluorography was performed as reported (Hanewinkel et al., 1987; Mach et al., 1992). Bands nonspecifically precipitated by the antisera used were identified in control experiments using antigen-deficient mouse embryonic fibroblasts. The quantity of individual labeled polypeptides was estimated by densitometric analysis of exposed films using ImageQuaNT v4.2 software (Molecular Dynamics, Sunnyvale, CA). The raw data were corrected for the number of methionine residues present in each polypeptide (see http://merops.sanger.ac.uk/ for the sequences of mouse procathepsin B, D and L and their processing products). The fraction of secreted proenzyme is expressed as percentage of the sum of intracellular and secreted antigen present.

Lysosomal enzyme secretion studies

Confluent cell monolayers (~10^7 cells) were incubated for 24 hours at 37°C in 10 ml of the respective culture medium containing 10% fetal bovine serum heat-inactivated for 30 minutes at 70°C (to inactivate any β-*N*-acetylhexosaminidase activity) and 10 mM NH_4Cl. Control cultures were incubated without addition of NH_4Cl. The supernatants were then aspirated and cleared by centrifugation (5 minutes, 320 ***g***) and subsequent passage through 0.22-μm filters. The cell monolayers were scraped into 500 μl of 20 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and ultrasonicated, followed by addition of 0.1% Triton X-100 and incubation for 30 minutes at 0°C. After centrifugation (10 minutes, 15,000 ***g***), the β-*N*-acetylhexosaminidase activity of cell extracts and conditioned media was determined spectrofluorimetrically with 4-methylumbelliferyl-β-*N*-acetylglucosaminide as substrate (Storrie and Madden, 1990). The fraction of secreted β-*N*-acetylhexosaminidase is expressed as percentage of the sum of intracellular and secreted enzyme present.

Preparation of microsomal protein extracts

For microsomal extracts, confluent cell monolayers (~10^7 cells) were scraped into 1 ml of 3 mM imidazole buffer (pH 7.4) containing 0.25 M sucrose, gently lysed with a Dounce homogenizer and then centrifuged (5 minutes at 400 ***g***). Microsomes were then pelleted (60 minutes at 105,000 ***g***) and extracted for 30 minutes at 0°C with 500 μl of 0.1 M sodium phosphate buffer (pH 6.0) containing 2 mM EDTA and 0.1% Triton X-100. After centrifugation (10 minutes at 15,000 ***g***), the supernatants were subjected to immunoblot analysis.

Subcellular fractionation

SCC-VII cells were incubated in complete culture medium in the absence or presence of either 10 mM NH_4Cl or 1 μM monensin for 10 hours at 37°C. Post-nuclear supernatants were obtained and fractionated by density-gradient centrifugation in 18% (v/v) Percoll gradients (initial density: 1.055 g/ml) as described (Schmid et al., 1999). The gradients were divided into ten fractions that were then analyzed for marker enzyme activities. The activity of the lysosomal marker β-*N*-acetylhexosaminidase was determined spectrofluorimetrically with 4-methyl-umbelliferyl-β-*N*-acetylglucosaminide (Storrie and Madden, 1990). The activity of the Golgi enzyme galactosyltransferase was measured with UDP-[^{3}H]galactose and chicken ovalbumin (Sigma) as described by Rome et al. (Rome et al., 1979) with minor modifications. The distribution of the endoplasmic reticulum (ER) marker NADH-cytochrome-*c*-reductase was determined in 75 mM potassium phosphate buffer (pH 7.5) containing 1 mM potassium cyanide, using 0.1 mg/ml NADH (disodium salt) and 20 μM cytochrome *c* (Sigma) as substrates. The increase of the optical density at 550 nm was then continuously monitored at room temperature. The buoyant density of each gradient fraction was determined either gravimetrically (Schmid et al., 1999) or with a refractometer. For detection of cathepsins D and L, fractions 1-3 (heavy fraction), 4-7 (intermediate fraction) and 8-10 (light fraction) were pooled and treated with 0.1% Triton X-100 for 30 minutes at 0°C. Residual Percoll was then removed by ultracentrifugation for 2 hours at 105,000 ***g***. Proteins were precipitated with 10% (w/v) trichloroacetic acid and 0.04% (w/v) sodium deoxycholate (30 minutes, 0°C) and recovered by centrifugation (10 minutes at 15,000 ***g***). The pellets were washed with ethanol and acetone, air-dried, dissolved in SDS-PAGE sample buffer and then subjected to SDS-PAGE and immunoblot analysis.

Immunoblot analysis

SDS-PAGE was performed in 12.5% gels run under reducing conditions. Separated polypeptides were electrophoretically transferred onto a nitrocellulose membrane (Hybond-C, Amersham Biosciences) as reported (Mach et al., 1992). After probing the membrane with the respective primary rabbit antibodies, bound immunoglobulins were visualized with goat anti-rabbit IgG immunoglobulins conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA) and chemiluminescence-based detection reagents (Coulibaly et al., 1999). Densitometric analysis of immunoblots was done using ImageQuaNT v4.2 software.

Analysis of phosphorylated oligosaccharides

Confluent cell monolayers were metabolically labeled with [^{3}H]mannose (1 mCi/ml) in 5 ml glucose-poor culture medium (1 mM glucose) for 8 hours at 37°C in the absence (control) or presence of 10 mM NH_4Cl or 1 μM monensin. Medium proteins were precipitated with 0.5 g/ml ammonium sulfate, redissolved in 2 ml 20 mM sodium acetate buffer (pH 5.5) containing 5 mM sodium β-glycerophosphate, and dialyzed against the same buffer. The retentate was concentrated by ultrafiltration (10 kDa cut-off) and incubated with 5 mU endo-β-*N*-acetylglucosaminidase H (Roche) as described (Dong and Sahagian, 1990). ^{3}H-labeled oligosaccharides thus released were then isolated by ultrafiltration as above. The ultrafiltrate was sequentially passed through a minicolumn filled with Dowex 50×8 cation-exchange resin (Sigma) and a Sep-Pak C_{18} reverse-phase cartridge (Waters, Milford, MA), both eluted with water. The unbound fraction was lyophilized and then desalted on a 1.5×7 cm column of Sephadex G-10 (Amersham Biosciences) eluted with water, and finally fractionated on diethyl-(2-hydroxypropyl)-aminoethyl-Sephadex (Sigma) according to Dong and Sahagian (Dong and Sahagian, 1990). To remove phosphomonoester groups, ^{3}H-labeled oligosaccharides were treated with 10 U alkaline phosphatase from calf intestine (Sigma) in 0.1 M Tris-HCl buffer, pH 8.0, for 1 hour at 37°C. Phosphodiester linkages were cleaved by incubation in 2 M acetic acid for 2 hours at 80°C. Sialic acid residues were removed by incubation with 5 mU *Arthrobacter ureafaciens* sialidase (Roche) in 0.1 M sodium acetate buffer (pH 5.5) for 16 hours at 37°C.

Immunofluorescence staining

SCC-VII cells grown on glass coverslips were treated in the absence or presence of either 10 mM NH_4Cl or 1 μM monensin in complete culture medium for 10 hours at 37°C. The cells were then fixed for 10 minutes in 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS). After blocking with PBS containing 2 mg/ml bovine serum albumin for 1 hour, the cells were incubated for 1 hour with the following antibodies diluted with PBS containing 0.1% saponin: rabbit anti-mouse cathepsin D (1:100), rabbit anti-bovine M6P/IGF2R (1:100), rabbit anti-mouse MPR46 (1:100), rat anti-mouse LAMP-1 (1:100), sheep anti-rat TGN38 (1 μg/ml). After a second blocking step in PBS containing 0.1% saponin and 5% fetal bovine serum (1 hour), bound primary antibodies were detected by incubation for 1 hour with the respective FITC- or Cy3-conjugated affinity-purified secondary antibodies (Jackson ImmunoResearch) at 5 μg/ml in PBS containing 0.1% saponin. All steps

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were performed at room temperature. The immunostained cells were examined using a Leica TCS SP2 confocal laser-scanning microscope equipped with diode, Ar and He/Ne lasers. Images from the confocal system were imported into Adobe Photoshop 5.5 for coloration.

Staining of acidic organelles with LysoSensor Yellow/Blue

SCC-VII cells and L-M(TK$^-$) fibroblasts grown on glass coverslips were treated in the absence or presence of either 10 mM NH_4Cl or 1 μM monensin in complete culture medium for 2 hours at 37°C. The cells were quickly rinsed and then incubated in PBS (supplemented with NH_4Cl and monensin where appropriate) containing 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for 10 minutes at 37°C before addition of 5 μM LysoSensor Yellow/Blue DND-160 (Molecular Probes, Eugene, OR). Cells were incubated for an additional 5 minutes at 37°C, then briefly rinsed with PBS and immediately observed by confocal laser-scanning microscopy (see above). Upon excitation at 405 nm, fluorescence emitted in the range of 420-500 nm (DAPI) and 500-580 nm (LysoSensor) was recorded simultaneously. The acquired images were then imported into Adobe Photoshop 5.5 for overlay and pseudocoloration.

Protein assay

Total protein was determined by the Bradford method using the Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA), using bovine serum albumin (Sigma) as a standard.

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