Functional characterization of wild-type and mutant human sialin

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The modification of cell surface lipids or proteins with sialic acid is essential for many biological processes and several diseases are caused by defective sialic acid metabolism. Sialic acids cleaved off from degraded sialoglycoconjugates are exported from lysosomes by a membrane transporter, named sialin, which is defective in two allelic inherited diseases: infantile sialic acid storage disease (ISSD) and Salla disease. To develop a functional assay of human sialin, we redirected the protein to the plasma membrane by mutating a dileucine-based internalization motif. Cells expressing the plasmalemmal construct accumulated neuraminic acid at acidic pH by a process equivalent to lysosomal efflux. The assay was used to determine how pathogenic mutations affect transport. Interestingly, while two missense mutations and one small, in-frame deletion associated with ISSD abolished transport, the mutation causing Salla disease (R39C) slowed down, but did not stop, the transport cycle, thus explaining why the latter disorder is less severe. Since neurological symptoms predominate in Salla disease, our results suggest that sialin is rate-limiting to specific sialic acid-dependent processes of the nervous system.

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Introduction

Diverse biological processes such as cell adhesion, signalling and virus infection depend on the modification of cell surface macromolecules with a group of nine-carbon monosaccharides known as sialic acids (Varki, 1999). Owing to their negative charge and terminal position in oligosaccharide chains, these sugars are accessible to specific lectins from apposing membranes. In vertebrates, many sialic acid-dependent interactions involve the families of selectins and sialic acid-binding immunoglobulin-like lectins (siglec), which, respectively, mediate adhesion between circulating lymphocytes and blood vessels (Patel *et al*, 2002) or regulate inter-

cellular signalling in the haemopoietic, immune and nervous system (Crocker, 2002). Sialic acids can also provide a physical barrier to other recognition mechanisms. For instance, polysialylation of the neural cell adhesion molecule (NCAM) prevents its homophilic binding and promotes plasticity during central nervous system (CNS) development (Kiss and Rougon, 1997).

The biological importance of sialylation is demonstrated by the existence of several disorders with altered sialic acid metabolism. For instance, sialyltransferases, which transfer sialic acid to nascent oligosaccharides in the Golgi, often show altered expression in cancer (Varki, 1999). Mutations in the rate-limiting enzyme of sialic acid synthesis, UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (GNE), cause two types of inherited disease: those that impair enzymatic activity cause recessive distal myopathies (Nonaka, 1999), whereas other missense mutations which suppress a negative allosteric mechanism of GNE cause sialic acid accumulation in the cytosol and a dominant form of sialuria (OMIM 269921) (Aula and Gahl, 2001). Degradation of sialoglycoconjugates is important as well. Diverse lysosomal storage diseases, such as sialidosis, Tay-Sachs disease or Sandhoff disease, are blocked at a specific enzymatic step of sialoglycoconjugate catabolism (Platt and Walkley, 2004). In galactosialidosis, two enzymes, lysosomal neuraminidase and β -galactosidase, are simultaneously affected because they cannot associate with their missing partner, protective protein/cathepsin A (van der Spoel *et al*, 1998). Finally, two recessive allelic diseases, Salla disease (OMIM 604369) and infantile sialic acid storage disease (ISSD; OMIM 269920), result from defective free sialic acid transport from lysosomes (Renlund *et al*, 1986; Mancini *et al*, 1991; Verheijen *et al*, 1999; Aula and Gahl, 2001).

ISSD is a very rare worldwide disease clinically characterized by facial dysmorphism, hepatosplenomegaly, failure to thrive, hypotonia and early death (<2 years). Salla disease, which prevails in Finland, primarily affects the CNS with limited somatic findings and near-normal lifespan (Aula and Gahl, 2001). Patients present with hypotonia, ataxia and delayed motor development during the first year. Speech development is impaired in childhood and all adult patients show severe mental retardation. Neuropathological (Autio-Harmainen *et al*, 1988) and magnetic resonance imaging studies (Haataja *et al*, 1994; Sonninen *et al*, 1999) revealed defective myelination in the CNS.

Both diseases are characterized by the presence of swollen lysosomes accumulating free sialic acid, and biochemical experiments on patient cultured cells demonstrated a lack of sialic acid transport across the lysosomal membrane (Aula and Gahl, 2001). Consistently, the causative gene, *SLC17A5*, was found to encode a novel transmembrane protein, sialin, showing homology to known secondary active transporters (Verheijen *et al*, 1999) such as the synaptic vesicle glutamate transporters and the renal phosphate transporter NaPi-1—for a review, see Reimer and Edwards

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(2004). In all, 20 pathogenic mutations of the *SLC17A5* gene have been described in the literature (Verheijen *et al*, 1999; Aula *et al*, 2000). A substitution of a conserved arginine, R39C, is present in the homozygous or heterozygous state in all known cases of Salla disease, but absent from ISSD. Most Salla disease patients are homozygous for this mutation, while the rarer cases of compound heterozygosity are generally more severely affected (Aula *et al*, 2000; Biancheri *et al*, 2002; Varho *et al*, 2002; Kleta *et al*, 2003). On the other hand, diverse mutations including large deletions and premature stops, but also missense mutations and a small in-frame deletion (Δ SSLRN), are observed in ISSD alleles (Verheijen *et al*, 1999; Aula *et al*, 2000).

Why different *SLC17A5* mutations cause different diseases is currently unknown. Transport measurements on patient lysosomes did not detect significant activity in both diseases (Renlund *et al*, 1986; Tietze *et al*, 1989; Mancini *et al*, 1991). A recent study revealed that the R39C mutation and, to a higher extent, the Δ SSLRN deletion retain sialin in the Golgi and hence reduce its lysosomal localization (Aula *et al*, 2002). However, it is unknown whether these mutations affect the transport activity, because sialin has been characterized at biochemical (Mancini *et al*, 1989, 1991; Havelaar *et al*, 1998), but not at the molecular, level. In this study, we addressed this issue using a novel approach based on the relocation of recombinant sialin to the plasma membrane by mutagenesis of a sorting motif. The advantage of this approach, which has been successfully applied to the lysosomal cystine transporter cystinosin (Kalatzis *et al*, 2001), is to create the equivalent of an 'inside-out lysosome' in which the poorly accessible, outward-directed organelle transport is studied as a classical cellular uptake. Interestingly, we found significant functional differences between ISSD- and Salla disease-associated mutants.

Results

Identification of a dileucine-based sorting motif

The late endosomal and lysosomal localization of sialin has been recently shown by cell fractionation and immunofluorescence studies (Aula *et al*, 2002). In order to identify the signals responsible for this localization, we mutated potential sorting motifs in tagged constructs of human sialin. The protein was fused either at its amino-terminus to GFP or at its carboxy-terminus to a V5 epitope. When transiently expressed in HeLa cells, both constructs showed a punctate intracellular distribution, which overlapped with the endogenous late endosomal and lysosomal protein LAMP1 by confocal microscopy (Figure 1A and C). Consistently, when co-expressed, the V5- and GFP-tagged proteins had identical distributions (Figure 1B). These data show that the GFP or V5 tagging do not affect the intracellular localization of sialin. For practical reasons, we used GFP tags throughout this study.

Lysosomal sorting signals generally consist of short sequence motifs recognized by cytosolic membrane coats (Bonifacino and Traub, 2003). Sequences corresponding to potential dileucine-based signals are found in the amino-terminal region (DRTPLL) and in the loops connecting the fourth and fifth (ERSKLL) and the sixth and seventh (EKEYIL) transmembrane domains of sialin. These sequences are cytosolic in the predicted topological model (Figure 2A). To

Figure 1 Intracellular localization of GFP- and V5-tagged sialin. Confocal sections of HeLa cells transiently expressing WT GFP-sialin (**A**, **B**) and/or sialin-V5 (**B**, **C**) show that the recombinant proteins colocalize extensively with each other and with the endogenous late endosomal and lysosomal marker LAMP1. Scale bar, 20 μ m.

examine whether they act as sorting signals, their critical leucine or isoleucine residues were mutated to alanine. Mutations in the second and third sequences concentrated GFP-sialin to a perinuclear pole, but preserved its punctate, intracellular appearance (Figure 2B). In contrast, when the first sequence was mutated, GFP-sialin displayed a diffuse membrane localization corresponding to the plasma membrane. A similar effect was observed when the first pair of leucine was mutated to glycine (see Figure 5A), when the whole DRTPLL sequence was deleted (data not shown) or when the mutated constructs were expressed in other cell lines (COS-7, human embryo kidney 293 (HEK 293)).

To confirm the plasmalemmal localization of the DRTPLL mutants, we performed surface biotinylation experiments in HEK 293 cells. Biotinylated, plasmalemmal proteins were separated from other cellular components by streptavidin affinity chromatography and the relative amounts of GFP-sialin in the intracellular and plasmalemmal pools were assessed by immunoblotting with anti-GFP antibodies. A single, diffuse band centred at 80 kDa was observed in GFP-sialin transfected cells (Figure 3), in agreement with the molecular weights of human sialin (54.6 kDa) and GFP (26.9 kDa). Its heterogeneity may originate from glycosylation, as several N-glycosylation sites are predicted in the sialin sequence. As illustrated in Figure 3, lanes 4–6, most GFP-sialin was intracellular when the wild-type (WT) protein was expressed. In contrast, the amount of GFP-sialin in the plasmalemmal pool dramatically increased when leucine residues 22 and 23 were mutated to glycine, in agreement with the fluorescence microscopy analysis (compare lanes 6 and 9).

These biochemical and morphological data demonstrate that the DRTPLL motif is necessary to target sialin to its intracellular destination.

Figure 2 Role of the DRTPLL motif in intracellular targeting of sialin. (A) Topological model of sialin showing potential dileucine-based sorting signals, pathogenic mutations and potential N-glycosylation sites. (B) The intracellular distribution of WT GFP-sialin and dileucine mutants in transiently transfected HeLa cells was analysed by epifluorescence microscopy. (C) Schematic representation of the CD4 chimeras. From top to bottom: CD4ΔCt, CD4-Nsia and CD4-Nsia L22G/L23G. (D) Steady-state localization of the CD4 constructs. Cells were fixed, permeabilized and incubated with SIM.2 monoclonal antibodies. CD4-Nsia is found on intracellular compartments, whereas the other constructs are mainly at the plasma membrane. Scale bars, 20 μm.

The DRTPLL motif acts as an internalization signal

Lysosomal membrane proteins reach their destination by two intracellular routes: a direct route, in which proteins are diverted to endosomes at the trans-Golgi network (TGN), and an indirect, endocytic route, in which proteins traffic to

Figure 3 Mutation of the DRTPLL motif results in cell surface expression. HEK293 cells transiently expressing GFP (lanes 1–3), WT GFP-sialin (lanes 4–6) or GFP-sialin L22G/L23G (lanes 7–9) were treated with an impermeant biotinylation reagent. After cell lysis, biotinylated proteins were purified on streptavidin-agarose beads. In lanes 10–12, biotinylation was omitted to verify the selectivity of the affinity chromatography. Equal amounts of cell lysate (tot) and streptavidin-unbound material (ub) were analysed by SDS-PAGE and immunoblotting using anti-GFP antibodies. Streptavidin-bound proteins (bo) were derived from an 18-fold higher amount of material. The position and molecular mass (kDa) of protein standards is shown on the left.

endosomes and lysosomes via the plasma membrane (Bonifacino and Traub, 2003). The relative importance of these routes for a specific protein depends on the ability of its sorting signals to be recognized at the TGN or at the plasma membrane. To examine whether the DRTPLL motif is recognized at the plasma membrane, we tested its capacity to trigger internalization of a resident plasma membrane protein.

We first constructed CD4-sialin chimera by substituting the amino-terminal region of sialin (amino acids 2–41) for the cytosolic, carboxy-terminal domain of the CD4 protein (Figure 2C), and analysed their steady-state localization in HeLa cells (Figure 2D). As reported previously (Garrido *et al*, 2001), the truncated CD4 lacking its cytosolic domain localized to the plasma membrane. In contrast, the chimera with an intact DRTPLL motif displayed a punctate intracellular distribution, while the chimera bearing a L22G/L23G mutation in the sialin domain remained at the plasma membrane.

We next tested the capacity of CD4-sialin chimeras to internalize antibodies bound to their extracellular domain. Transiently transfected HeLa cells were pre-labelled with antibodies at 4°C and incubated at 37°C for increasing durations. Cells expressing the truncated CD4 or CD4-sialin L22G/L23G proteins displayed a membrane labelling that did not evolve over time and disappeared when cells were washed with an acidic medium (Supplementary Figure 1A and B). In contrast, antibodies bound to the WT CD4-sialin chimera rapidly moved to punctate structures, which resisted extracellular acid washing. The DRTPLL motif is thus an internalization signal. Interestingly, most antibodies internalized by the CD4-sialin chimera reached late endosomal compartments, since the corresponding puncta were positive for GFP-sialin when both constructs were expressed in HeLa cells (Supplementary Figure 1C). The DRTPLL motif thus seems sufficient to target sialin to its final destination.

Characterization of the transport activity of human sialin

The molecular function of sialin has been inferred from the biochemical defect of sialic acid storage diseases; however, it has not been directly studied. To develop a functional assay of sialin, we took advantage of the cell surface localization of the DRTPLL mutants and examined their capacity to translocate sialic acid across the plasma membrane. This approach allows to study lysosomal efflux as a classical cellular uptake (Kalatzis *et al*, 2001).

HEK 293 cells were transiently transfected with GFP-sialin plasmids and analysed for their ability to take up *N*-acetyl-[6-³H]neuraminic acid ([³H]Neu5Ac) from the extracellular medium. When uptake was performed at neutral pH, cells expressing GFP-sialin L22G/L23G accumulated [³H]Neu5Ac as much as mock-transfected cells (Figure 4A). However, uptake was strongly activated when the extracellular medium (topologically equivalent to the lysosomal lumen in our assay) was acidified to a pH value of 5.6. A mean increase of 5.5 ± 0.5 -fold ($\pm$ standard error of the mean (s.e.m.)) over background was obtained from 19 independent experiments. In contrast, cells expressing the WT construct took up sialic acid at background (mock) level at both pH. To examine whether the L22G/L23G sialin-mediated signal represented membrane transport or binding to the cell surface, cells were perforated with digitonin for 6 min after their incubation with [³H]Neu5Ac. As shown in Figure 4A, this treatment released the radioactivity. The relocalization of GFP-sialin to the cell surface is thus associated with an ability to transport sialic acid across the plasma membrane.

The sialin-mediated process remained linear for 15 min (data not shown). We thus used uptake periods ≤ 15 min throughout this study to measure transport velocities. When increasing [³H]Neu5Ac concentrations were used, uptake increased linearly up to 0.3 mM and progressively saturated for higher values (Figure 4B), with a K_M of 1.52 ± 0.26 mM at pH 5.6 (mean $\pm$ s.e.m. of four independent transfections; see Table I). The maximal velocity (V_{max}), which depends on the transfection efficiency, varied from 104 to 245 pmol/min per 10^6 cells, with a mean value of 174 pmol/min per 10^6 cells (Table I).

To characterize the dependence of sialin on protons, uptake was measured at several extracellular pH's between 7.4 and 5.0. As illustrated in Figure 4C, sialin-mediated uptake was low or undetectable at pH ≥ 6.5 , but increased linearly when pH decreased below 6.5. This stimulation of sialin at acidic pH might result from a higher affinity for protonated sialic acid, from an allosteric control by protonable residues exposed to the noncytosolic compartment, or from a co-transport of H^+ with sialic acid. To discriminate between these possibilities, we first studied the dependence of transport parameters on extracellular pH (Table I). Saturation kinetics experiments performed at pH 5.0 yielded a K_M value (2.13 ± 0.29 mM) similar to that obtained at pH 5.6, thus ruling out the hypothesis of a better affinity for protonated Neu5Ac. In contrast, the maximal velocity increased when pH decreased. To avoid variations due to differences in transfection efficiency, we performed one experiment at both pH 5.6 and 5.0 and observed a 1.6-fold increase in V_{max} (K_M values: 2.16 and 1.93 mM, respectively), in agreement with the pH dependence curve obtained at nonsaturating Neu5Ac concentration (Figure 4C).

Figure 4 Sialin mediates a pH gradient-driven sialic acid transport. (A) HEK 293 cells expressing sialin at the cell surface (GFP-sialin L22G/L23G construct) take up [³H]Neu5Ac from acidic (pH 5.6, filled bars), but not from neutral (pH 7.4, open bars) extracellular medium. Perforation of the plasma membrane with 20 μ M digitonin released the radioactivity accumulated into GFP-sialin L22G/L23G-transfected cells (striped bar). (B) Saturation kinetics of sialin-mediated transport. Mock- (□) and GFP-sialin L22G/L23G-transfected (■) cells were incubated for 15 min with increasing concentrations of Neu5Ac at pH 5.6. Nonlinear regression yielded K_M and V_{max} values of 2.16 ± 0.16 mM and 245 ± 9.7 pmol/min per 10^6 cells in this experiment. (C) [³H]Neu5Ac uptake by mock- (open symbols) and GFP-sialin L22G/L23G-transfected (filled symbols) cells was performed in MES (squares) or MOPS (triangles) buffer adjusted to several pH values. Transport was stimulated at acidic pH. (D) At pH 5.6, dissipation of the transmembrane pH gradient by 5 μ M nigericin (striped bars) inhibited sialin L22G/L23G-mediated transport by more than 90%. For all experiments, error bars indicate s.e.m.

The remaining two hypotheses were assessed using the proton ionophore nigericin, which exchanges intracellular K^+ for extracellular H^+ and thus dissipates the pH gradient

Table I Kinetic transport parameters of active sialin constructs

Protein	pH	K_M (mM)	V_{max}		<i>n</i>
			pmol/min per 10^6 cells	Relative to WT	
Control	5.6	1.52 ± 0.26	174 ± 35	—	4
Control	5.0	2.13 ± 0.29	696 ± 155	1	3
R39C	5.0	2.61 ± 1.60	226 ± 116	0.29 ± 0.11	3
K136E	5.0	1.39 ± 0.37	191 ± 48	0.28 ± 0.04	3

HEK 293 cells expressing GFP-sialin L22G/L23G without (control) or with pathogenic mutations were analysed for [3 H]Neu5Ac uptake at pH 5.0 or 5.6. Kinetic parameters were determined by nonlinear regression using the SigmaPlot software. Values are means $\pm$ s.e.m. of *n* independent experiments.

Table II Substrate selectivity of sialin-mediated transport

Inhibitor	Sialin L22G/L23G-mediated transport (% activity)	Mancini <i>et al</i> (1989)	Havelaar <i>et al</i> (1998)
Neu5Ac	37 ± 6 (<i>n</i> = 4)	10	0
D-Glucuronate	21 ± 3 (<i>n</i> = 5)	10	0
D-Gluconate	31 ± 4 (<i>n</i> = 5)	14	ND
N-acetyl-D-glucosamine	102 ± 17 (<i>n</i> = 3)	100	ND
N-acetyl-D-mannosamine	101 ± 13 (<i>n</i> = 3)	100	ND
Pyruvate	35 ± 4 (<i>n</i> = 3)	50	24
L-Lactate	26 ± 3 (<i>n</i> = 5)	ND	8
Succinate	79 ± 5 (<i>n</i> = 5)	ND	0
Maleate	91 ± 3 (<i>n</i> = 5)	ND	26
L-Glutamate	98 ± 8 (<i>n</i> = 4)	86	107
L-Aspartate	99 ± 13 (<i>n</i> = 4)	89	ND

Inhibitors (7 mM) were added simultaneously to [3 H]Neu5Ac at pH 5.6. Results, expressed as % of activity in the absence of inhibitor, are means $\pm$ s.e.m. of *n* independent observations. For comparison, results of similar *cis*-inhibition experiments performed on rat liver lysosomal membranes (Mancini *et al*, 1989) or on purified proteoliposomes (Havelaar *et al*, 1998) are shown on the right. ND, not determined.

across the plasma membrane. As illustrated in Figure 4D, this treatment abolished Neu5Ac uptake. Therefore, a transmembrane pH gradient (rather than a mere acidic extracellular pH) is required to drive sialin, in agreement with a H^+ /sialic acid symport mechanism.

We also determined the substrate selectivity of human sialin by adding unlabelled compounds (7 mM) simultaneously to [3 H]Neu5Ac in the transport assay. As shown in Table II, acidic sugars such as gluconic acid or D-glucuronic acid inhibited uptake as efficiently as, or better than, Neu5Ac. In contrast, neutral sugars such as N-acetylmannosamine or N-acetylglucosamine had no effect. We also tested aliphatic monocarboxylates and dicarboxylates, since these compounds were reported to compete with sialic acid for transport across rat liver lysosomal membranes (Havelaar *et al*, 1998, 1999). Monocarboxylates such as L-lactate or pyruvate inhibited uptake as efficiently as acidic sugars. However, aliphatic dicarboxylates such as maleate or succinate did not significantly inhibit sialin-mediated transport, in contrast to the aforementioned studies. L-Glutamate, which is transported by three paralogs of sialin (Reimer and Edwards, 2004), and L-aspartate had no effect.

In conclusion, sialin is a H^+ -coupled transporter able to export sialic acid and other acidic monosaccharides out of the lysosome, as anticipated from previous studies on sialic acid storage diseases.

Effect of pathogenic mutations on sialic acid transport

We then used the functional assay of sialin to characterize the molecular effect of mutations causing sialic acid storage disease (Verheijen *et al*, 1999; Aula *et al*, 2000). Only muta-

tions that do not preclude synthesis of a full-length polypeptide were considered (Figure 2A): the R39C mutation, which is responsible for Salla disease; three missense mutations (H183R, P334R, G371V) and one short, in-frame deletion (SSLRN268–272del, referred to as Δ SSLRN hereafter) found in individuals with ISSD; and one missense mutation, K136E, found in a compound-heterozygote individual with a severe form of Salla disease (in addition to the R39C allele).

In preliminary experiments, we examined whether GFP-sialin L22G/L23G was still expressed at the plasma membrane after introducing these pathogenic mutations. As illustrated in Figure 5A, this held true for all mutations but G371V, which was retained in the endoplasmic reticulum (see Supplementary Figure 2) and thus was not investigated further. The remaining mutations were analysed for an effect on transport by expressing GFP-sialin L22G/L23G constructs in HEK 293 cells. The assay was performed at pH 5.0 to increase its sensitivity. As illustrated in Figure 5B, all mutations associated to ISSD (H183R, P334R, Δ SSLRN) abolished transport. In contrast, the R39C mutant showed positive, yet reduced, transport activity. This was also true for the K136E mutation, with a milder reduction. The residual activities observed in these conditions represented 17 ± 2 and $55 \pm 6\%$ of control (GFP-sialin L22G/L23G without pathogenic mutation; three independent experiments). Since fluorescence microscopy might have eluded quantitative variations in cell surface expression, we also performed surface biotinylation experiments and quantitative immunoblotting analysis to measure the relative levels of plasmalemmal sialin. As shown in Figure 5C, similar levels were observed for the control and mutant proteins. Values of 99.8 ± 8.8 and

Figure 5 Effect of pathogenic mutations on sialin activity. (A) The R39C, P334R, H183R, Δ SSLRN and K136E mutations did not impair surface expression of GFP-sialin L22G/L23G in HeLa cells. Scale bar, 20 μ m. (B) HEK 293 cells expressing GFP-sialin L22G/L23G without (control) or with pathogenic mutations were analysed for [³H]Neu5Ac uptake at pH 7.4 (open bars) or 5.0 (filled bars). Only the R39C and K136E mutants showed partial activity. (C) Surface biotinylation of transfected HEK 293 cells and quantitative immunoblotting analysis revealed similar amounts of GFP-sialin L22G/L23G at the cell surface, whatever the pathogenic mutation. The number of independent measurements is indicated in brackets.

122 $\pm$ 3.9% of control were obtained for the R39C and K136E mutants (four independent experiments). Therefore, the lack or decrease of activity in the mutants reflects an impaired ability to transport sialic acid.

To characterize further the R39C and K136E mutants, we determined their kinetic parameters (Table I). The R39C mutation did not alter the K_M , but induced a 3.4-fold reduction in V_{max} relative to control. K136E induced an identical (3.6-fold) reduction in V_{max} and it slightly increased the affinity for Neu5Ac, thus explaining the higher divergence from R39C at low substrate concentration (Figure 5B). Therefore, both mutants are mainly characterized by a slower turnover of the transport cycle.

Effect of pathogenic mutations on localization

Since the R39C and K136E mutants are partially active, we asked whether they are present on late endosomes and lysosomes in the absence of L22G/L23G mutation. We thus introduced pathogenic mutations into GFP-sialin (with an intact DRTPLL motif) and examined the localization of the protein in HeLa cells. For comparison, ISSD-associated mutations were also investigated. Results are shown in Figure 6. In most cases (R39C, Δ SSLRN, H183R, K136E), the mutant was found on LAMP1-negative puncta and at a juxtanuclear compartment resembling the Golgi, in addition to LAMP1-positive late endosomes and lysosomes. In agreement with a previous study (Aula *et al*, 2002), a milder mislocalization was observed for the R39C mutant. In contrast, sialin localization was not affected by the P334R mutation since, as the WT sialin P334R fully overlapped with LAMP1.

Discussion

The role of sialin in lysosomal efflux of acidic sugars has been inferred from the biochemical and genetical defects of sialic acid storage diseases (Renlund *et al*, 1986; Tietze *et al*, 1989; Mancini *et al*, 1991) and from its sequence homology to other secondary active transporters (Verheijen *et al*, 1999). In this study, we provide direct evidence of its transport activity, using a whole-cell assay based on sialin missorting, and we show that the transport activity of mutant sialin correlates with the existence of two clinical forms of sialic acid storage disease.

Activity and targeting of the WT protein

Our study identified a dileucine-based sorting motif, DRTPLL, which is critical to the lysosomal and late endosomal localization of sialin. When the motif is mutated, sialin is massively redirected to the plasma membrane (Figures 2 and 3) and, conversely, its introduction into an unrelated plasma-membral protein triggered internalization and delivery to late endosomes (Supplementary Figure 1). The motif thus plays a major role in sialin trafficking. It follows the consensus sequence [DE]XXXL[LI] and thus may bind one or several of the heterotetrameric adaptor protein complexes (AP-1–AP-4) of coat sorting machineries (Bonifacino and Traub, 2003). Its position in the amino- rather than the carboxy-terminal region of the polypeptide is unusual, but not unique, as VAMP4, the insulin-regulated aminopeptidase and the invariant chain of class II MHC proteins show similar localization.

The fact that the DRTPLL motif is recognized at the plasma membrane (Supplementary Figure 1) implies that sialin can reach lysosomes and late endosomes via an indirect, endocytic route. This route may represent the main pathway followed by sialin. However, since our experiments do not exclude DRTPLL recognition at the TGN in addition to the

Figure 6 Effect of pathogenic mutations on sialin intracellular localization. HeLa cells transiently expressing WT or mutant (R39C, Δ SSLRN, K136E, H183R and P334R) GFP-sialin with an intact DRTPLL motif were fixed, stained with anti-LAMP1 antibodies and analysed by epifluorescence microscopy. GFP-sialin, LAMP1 and merged images (sialin, green; LAMP1, red) are shown from left to right. The WT and P334R proteins fully colocalize with LAMP1. Other mutants partially colocalize with LAMP1 (arrowheads), but they are also found in a perinuclear compartment and in LAMP1-negative puncta (arrows). Images are 3D reconstructions of the cells after deconvolution. Insets are magnifications of the rectangles shown on merged images. Scale bar, 20 μ m.

plasma membrane, the endocytic route may just represent a salvage pathway for a direct, intracellular route. This issue and the possible involvement of other sorting signals need further investigation.

Thus far, all knowledge on lysosomal sialic acid transport resulted from biochemical studies of rat liver lysosomal membranes (Mancini *et al*, 1989, 1991; Havelaar *et al*, 1999), of a 57-kDa protein purified from these membranes (Havelaar *et al*, 1998) or of human lysosomal membranes purified from cultured cells (Mancini *et al*, 1991). Our study now provides transport data at the molecular level. The activity of recombinant sialin is highly similar to that of lysosomal membranes, since both transports are driven by

a downhill H^+ gradient (Figure 4C and D) (Mancini *et al*, 1989, 1991; Havelaar *et al*, 1998) and both recognize acidic, but not neutral, monosaccharides as well as aliphatic monocarboxylates (Table II and references therein). The inhibition of human sialin by glucuronic acid is also consistent with its accumulation in lysosomes from Salla disease and ISSD patients (Blom *et al*, 1990).

Our data differ from previous biochemical studies in two minor points, however. First, the Neu5Ac affinity is six-fold lower for the recombinant human protein ($K_M = 1.52 \pm 0.26$ mM) than for rat membranes ($K_M = 0.24 \pm 0.07$ mM) (Mancini *et al*, 1989). This discrepancy should not reflect divergence between mammalian species, since D-glucuronic

acid had identical K_M values in rat and human membranes (Mancini *et al*, 1991; Havelaar *et al*, 1998). One possible explanation is that substrate affinity is decreased in our model because sialin is expressed at an ectopic membrane. Alternatively, the discrepancy may result from topological differences between lysosomal vesicles and our 'inside-out lysosome' model. Indeed, if sialin interacts asymmetrically with Neu5Ac, precisely, if its affinity is better from the cytosolic than from the luminal compartment, studies on randomly oriented vesicles would preferentially detect the process with the highest affinity (cytosol-to-lumen transport), while our assay is selective for the physiological (lumen-to-cytosol) one—for an example of kinetic asymmetry, see Reig *et al* (2002).

Another discrepancy concerns the effect of dicarboxylates. Havelaar *et al* (1998) reported that the purified rat transporter is strongly *cis*-inhibited by succinate and maleate, yet we did not observe any inhibition (Table II). The aforementioned topological differences might again explain this discrepancy, since dicarboxylates had no effect on membrane vesicles when added to the *trans*-compartment (Havelaar *et al*, 1998). It should be noted that the biological significance of sialin interaction with aliphatic mono- or dicarboxylates is unclear, because the lysosomal lumen is not expected to generate or import these compounds.

Molecular pathogenesis of free sialic acid storage: why two diseases?

Mutations in the sialin gene result in two diseases, which share a common biochemical defect (the accumulation of free sialic acid in lysosomes) but dramatically differ in their clinical course and symptoms: ISSD is an early fatal disorder, which displays many of the characteristic features of lysosomal disorders, whereas Salla disease is essentially a neurological disorder with near-normal lifespan (Aula and Gahl, 2001). To investigate the molecular basis of these differences, we examined how pathogenic mutations affect sialic acid transport.

We studied five missense mutations and one small in-frame deletion associated either to ISSD or to Salla disease (Verheijen *et al*, 1999; Aula *et al*, 2000). One mutation associated with ISSD, G371V, caused aggregation and degradation of the protein (Supplementary Figure 2). The remaining ISSD mutations (H183R, P334R, Δ SSLRN) did not impair expression, but abolished the capacity of the protein to translocate Neu5Ac (Figure 5). Therefore, all these mutations represent loss-of-function mutations, in agreement with the presence of frameshift mutations or large deletions in other ISSD alleles.

In contrast, the mutation responsible for Salla disease, R39C, decreased but did not abolish transport (Figure 5 and Table I), showing a correlation between the molecular and clinical phenotypes. Such a correlation has not been detected thus far, since transport studies on human lysosomes failed to detect any difference between Salla disease and ISSD (Renlund *et al*, 1986; Tietze *et al*, 1989; Mancini *et al*, 1991). Our whole-cell assay thus appears more sensitive than measurements on membrane vesicles. It should be noted that the residual activity of sialin R39C directly explains why Neu5Ac and glucuronic acid accumulate at a lower level in Salla disease than in ISSD (Blom *et al*, 1990; Aula and Gahl, 2001).

Pathogenic mutations also caused intracellular mislocalization (Figure 6), in agreement with a previous study (Aula *et al*, 2002). We observed sialin R39C at a perinuclear compartment identified as the Golgi (C Sagné, unpublished data), as well as in LAMP1-negative puncta, which might represent sialin molecules *en route* to the lysosome (Aula *et al* (2002), who blocked protein synthesis prior to analysis, did not observe these puncta). Δ SSLRN and H183R induced similar, but apparently stronger, mislocalization. It might be argued that mislocalized sialin, whatever its activity, has some toxic effect, which contributes to the higher severity of ISSD. However, this hypothesis is at variance with our observation that P334R, which abolishes transport and causes ISSD, does not alter intracellular localization. It is important to stress that the mutant proteins, including sialin R39C, partially localized to the lysosome (Figure 6) (Aula *et al*, 2002). Therefore, the residual activity of the Salla disease mutant implies positive, yet reduced, lysosomal function.

To explore the relationship between the molecular and clinical phenotypes in more detail, we also analysed a mutation (K136E) found in one Salla disease patient but not, to our knowledge, in ISSD (Aula *et al*, 2000). Clinical studies have distinguished two forms of Salla disease, which correlate to some extent with the genotype: whereas nearly all patients with the classical disease are homozygous for R39C, the rarer cases of 'severe' Salla disease are generally compound heterozygotes with the R39C mutation in one allele and a large deletion, a premature stop, Δ SSLRN or K136E in the other allele (Aula *et al*, 2000; Biancheri *et al*, 2002; Kleta *et al*, 2003). Interestingly, K136E reduced transport as much as R39C at high Neu5Ac concentration (Table I), that is in a situation of sialic acid storage, and it mislocalized sialin apparently more strongly than R39C (Figure 6). The combination of these effects might thus explain the higher severity of Salla disease in the R39C/K136E individual. This pathogenic scenario should be considered with caution, however, because there is still a wide phenotypic variation among R39C homozygotes (Varho *et al*, 2002).

Physiological implications

In contrast to ISSD, somatic findings such as hydrops fetalis, hepatosplenomegaly and dysostosis multiplex are absent from Salla disease. Growth retardation is limited and facial dysmorphism, when present, occurs at late stages (Aula and Gahl, 2001). The residual activity of sialin R39C thus seems sufficient for most peripheral tissues. On the other hand, the CNS is severely affected, with neurological signs such as hypotonia and ataxia within the first year, mental retardation and defective cerebral myelination (Haataja *et al*, 1994; Sonninen *et al*, 1999; Aula and Gahl, 2001). Therefore, our transport measurements suggest that sialin is rate-limiting to specific processes of the brain (it may be noted that, because V_{max} is decreased by R39C, sialic acid accumulation in Salla lysosomes cannot compensate for the reduced activity).

An attractive possibility for such sialin-dependent processes is the interaction of gangliosides with the sialic acid-binding lectin siglec-4, also known as myelin-associated glycoprotein (MAG), because it might explain the central dysmyelination observed in sialic acid storage disorders. Indeed, MAG is located at the periaxonal membrane of myelin (Trapp *et al*, 1989), it binds complex gangliosides of

the nerve cell membrane (Collins *et al*, 1999) and mice lacking either MAG (Schachner and Bartsch, 2000) or complex gangliosides (Sheikh *et al*, 1999) show selective CNS dysmyelination. Since MAG has a higher affinity for complex (highly sialylated) gangliosides (Collins *et al*, 1999), decreased sialin activity in the lysosome—or in nonlysosomal structures present in the axon (Aula *et al*, 2004)—could limit the availability of sialic acid in the cytosol, induce ganglioside hyposialylation and thus impair the axo-glial MAG/ganglioside interaction (see Keppler *et al* (1999) for a similar mechanism caused by decreased sialic acid biosynthesis). It will thus be important to analyse the sialylation level of brain glycoconjugates when an animal model of Salla disease is available.

Materials and methods

cDNA constructs

The IMAGE cDNA clone #3847279, which encodes a full-length human sialin, was obtained from the *Deutsches Ressourcenzentrum für Genomforschung* (RZPD). Its coding sequence is identical to that described by Verheijen *et al* (1999) (DDBJ/EMBL/GenBank accession number #AJ387747), except for a silent substitution at the wobble position of codon 82 (GCA instead of GCG). This silent substitution corresponds to a single-nucleotide polymorphism (refSNP ID #rs472294).

All cDNA modifications were performed by PCR using the primers listed in Supplementary Table I. Constructs were verified by automated sequencing over the whole coding sequence. In order to fuse the carboxy-terminus of sialin to a V5 epitope (GKPIPNLLGLDST), the coding sequence was amplified from the IMAGE cDNA using the primers SIA-7S and SIA-8A, and subcloned at the *HindIII* and *SacII* sites of the pcDNA3.1/V5-His-TOPO vector (Invitrogen). Fusion of the amino-terminus to GFP was performed by amplification with the primers SIA-24S and SIA-29A and subcloning at the *EcoRI* and *SacII* sites of the pEGFP-C2 vector (Clontech). Eleven mutations were introduced into the sialin coding sequence as described in Supplementary data.

A truncated construct of human CD4 (amino acids 1–396, designated as CD4ΔCt) was kindly provided by B Dargent (Garrido *et al*, 2001). The coding sequence was amplified using the CD4-1S and CD4-2A primers and the product was cloned at the *KpnI* and *NotI* sites of pEGFP-N1 (Clontech) in replacement of the GFP sequence. To construct CD4-sialin chimeras, a CD4 product amplified using the CD4-1S and CD4-4A primers was cloned at the *KpnI* and *BamHI* sites of pEGFP-N1. The sequence for the N-terminal domain of sialin, obtained using primers SIA-26S and SIA-27A, was then introduced at the *BamHI* and *NotI* sites in replacement of EGFP.

Cell culture and transfection

HeLa and HEK 293 cells were grown under 5% CO₂ in glucose-rich, Glutamax-I-containing Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 7.5% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

HeLa cells were transfected by electroporation using a GHT 1287 electropulsator (Jouan). Typically, 2×10^6 HeLa cells in 50 µl of ice-cold phosphate-buffered saline (PBS; pH 7.4) were mixed with 5 µg plasmid, immediately subjected to nine square pulses (200 V, 3 ms) delivered at 1 Hz by 4-mm-spaced electrodes, diluted with 6 ml of culture medium and distributed in 12 wells (15-mm diameter) of a 24-well culture plate. HEK 293 cells were plated (300 000 cells/well) into poly-D-lysine-coated 24-well plates and transfected on the following day with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol. HeLa and HEK293 cells were analysed 2 or 3 days after transfection.

Immunofluorescence analysis

Cells grown on glass coverslips were washed with PBS containing 100 µM MgCl₂ and 100 µM CaCl₂ (PBS/Ca/Mg), and fixed with 4% paraformaldehyde (PFA; Sigma) for 10 min. After washing and quenching PFA with 50 mM NH₄Cl for 15 min, cells were washed

with PBS and permeabilized in blocking buffer (0.05% saponin/0.2% BSA in PBS/Ca/Mg) for 20 min. Coverslips were then incubated for at least 1 h with primary antibodies in blocking buffer, washed with PBS, incubated for 1 h with secondary antibodies in the same buffer and rinsed with PBS and water. Coverslips were mounted on glass slides with Mowiol. All steps were performed at room temperature. The following antibodies were used at the indicated dilutions: mouse anti-V5-tag (Serotec) 1 µg/ml, rabbit anti-V5 (Chemicon) 0.2 µg/ml, mouse anti-LAMP1 (H4A3; Developmental Studies Hybridoma Bank, University of Iowa) 0.75 µg/ml, Cy3-conjugated donkey anti-mouse (Jackson ImmunoResearch) 1.4 µg/ml, Alexa Fluor 488-conjugated goat anti-rabbit (Molecular Probes) 4 µg/ml and Alexa Fluor 568-conjugated goat anti-mouse (Molecular Probes) 2 µg/ml.

Epifluorescence pictures were acquired under a $\times 100$ objective lens with a Leica DM RXA2 microscope equipped with a CCD camera (Coolsnap). For comparing the cellular distribution of two markers, pictures were either acquired with a spectral confocal Leica TCS/SP2 microscope or epifluorescence data were deconvoluted. Sections for deconvolution microscopy were collected throughout the cell using a z-axis focus drive with a spacing of 0.2 µm. Stacked images were deconvoluted using the PSF-based Iterative 3D Deconvolution module of the Metamorph software (Universal Imaging Corporation). All images were processed using Adobe Photoshop.

Endocytosis assay

HeLa cells transfected with CD4 constructs were briefly washed with ice-cold DMEM and incubated for 30 min at 4°C with the mouse monoclonal anti-CD4 antibodies SIM.2 or SIM.4 (NIH AIDS Research and Reference Reagent Program) diluted in DMEM at 65 and 25 ng/ml, respectively. Coverslips were then washed extensively with ice-cold PBS, transferred to 37°C-prewarmed DMEM and incubated for increasing periods of time in the air/CO₂ incubator. To remove SIM.4 antibodies bound to the cell surface (acid wash), cells were incubated for 5 min at 4°C in 0.5 M NaCl and 0.2 M acetic acid (pH 2.6). Cells were fixed with PFA and processed for immunofluorescence as described above.

Sialic acid transport

HEK 293 cells were washed twice with 500 µl of uptake buffer A (5 mM D-glucose, 140 mM NaCl, 1 mM MgSO₄, 20 mM K⁺-phosphate pH 7.4) and incubated for 15 min at room temperature in 200 µl of uptake buffer A or B (identical to buffer A, but adjusted to pH 5.6) containing 0.1 µCi N-acetyl-[6-³H]neuraminic acid (20 Ci/mmol; American Radiolabeled Chemicals). The reaction was stopped by aspiration of the uptake medium, followed by two brief washes with 500 µl of ice-cold uptake buffer at the corresponding pH. Cells were lysed in 200 µl 0.1 N NaOH and the accumulated radioactivity was counted in Emulsifier-Safe cocktail (Packard) using a Tri-Carb 2100 TR liquid scintillation analyzer (Packard). Transport was studied at other pH values by replacing phosphate by 10 mM MES or MOPS. Saturation kinetics experiments performed at pH 5.0 (MES buffer) were carried out with shorter incubations (6 min) to measure transport velocities. All compounds used for inhibition studies were obtained from Sigma. Measurements were carried out in triplicate and are expressed as means $\pm$ s.e.m. Each experiment was performed three or more times on independent transfections. Michaelis–Menten kinetic parameters were derived by nonlinear regression of untransformed data using the SigmaPlot 8.0 software (Systat Software, Inc.).

Western blot analysis

HEK 293 cells were chilled on ice, rinsed twice with PBS and scraped in 2 ml PBS containing protease inhibitors (5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin and 1 mM PMSF). Cells were centrifuged at 1000 g for 10 min at 4°C and pellets were immediately frozen in liquid nitrogen and stored at –20°C. Pellets were solubilized in Laemmli's sample buffer containing Benzonase (Merck) and the equivalent of 7×10^5 cells was loaded directly onto a 10% SDS-PAGE gel. The separated proteins were electrotransferred to a nitrocellulose membrane and, after blocking for 1 h in PBS containing 5% nonfat dry milk, the membrane was incubated for 1 h at room temperature with a 1:1000 dilution of mouse anti-GFP antibody (Roche Applied Science), washed three times in 0.05% Tween/PBS and incubated for 1 h at room temperature with a 1:100 000 dilution of horseradish peroxidase-conjugated

antibodies against mouse whole immunoglobulins (Jackson Immunoresearch). Immune complexes were detected using the Lumi-light^{PLUS} Western Blotting Substrate (Roche). When specified, the membrane was stripped and re-probed with an anti- β actin monoclonal antibody (clone AC-74, Sigma).

Quantitative Western blot analysis was performed using ¹²⁵I-labelled sheep anti-mouse Ig antibody (Amersham Biosciences) as secondary antibody. The membrane was incubated for 1 h in 0.05% Tween/PBS with a 1:500 dilution (0.2 μ Ci/ml) of radiolabelled antibody. After washing, the membrane was exposed overnight to a Storage Phosphor Screen (Kodak). After scanning with a Phosphor-imager 400E instrument (Molecular Dynamics), the signal associated to each immunoreactive band was determined using the ImageQuant software (Molecular Dynamics).

Cell surface biotinylation

At 2 days after transfection, 2×10^6 HEK293 cells were washed twice with ice-cold PBS/Ca/Mg and biotinylated for 30 min at 4°C using 1 mg/ml of the cell-impermeant, cleavable reagent sulpho-NHS-SS-biotin (Pierce) in PBS/Ca/Mg. Unbound biotin was quenched for 20 min at 4°C with 100 mM glycine in PBS/Ca/Mg. After two washes, cells were lysed for 1 h in 200 μ l lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 1% Triton X-100, 1 mM PMSF, 0.1 mM leupeptin, 0.1 μ M pepstatin A). The cell lysate (0.2 mg protein in 600 μ l) was clarified by sedimentation at 14 000 r.p.m. for 10 min and the supernatant was incubated for 2 h at 4°C with 50 μ l streptavidin-agarose beads (Sigma) under gentle

agitation. The beads were sedimented at 1000 r.p.m. for 30 s. The supernatant (unbound material) was recovered and beads were washed three times with 1 ml lysis buffer, once with 500 mM NaCl and 50 mM Tris-HCl (pH 7.5), and once with 10 mM Tris/HCl (pH 7.5). The bound material was eluted in 50 μ l Laemmli's sample buffer. The totality of bound proteins and an aliquot of unbound proteins or cell lysate (30 μ l from the total volume of 600 μ l) were resolved by SDS-PAGE and analysed by immunoblotting with anti-GFP antibodies.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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