Varied Mechanisms Underlie the Free Sialic Acid Storage Disorders*

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Salla disease and infantile sialic acid storage disorder are autosomal recessive neurodegenerative diseases characterized by loss of a lysosomal sialic acid transport activity and the resultant accumulation of free sialic acid in lysosomes. Genetic analysis of these diseases has identified several unique mutations in a single gene encoding a protein designated sialin (Verheijen, F. W., Verbeek, E., Aula, N., Beerens, C. E., Havelaar, A. C., Joosse, M., Peltonen, L., Aula, P., Galjaard, H., van der Spek, P. J., and Mancini, G. M. (1999) Nat. Genet. 23, 462–465; Aula, N., Salomaki, P., Timonen, R., Verheijen, F., Mancini, G., Mansson, J. E., Aula, P., and Peltonen, L. (2000) Am. J. Hum. Genet. 67, 832–840). From the biochemical phenotype of the diseases and the predicted polytopic structure of the protein, it has been suggested that sialin functions as a lysosomal sialic acid transporter. Here we directly demonstrate that this activity is mediated by sialin and that the recombinant protein has functional characteristics similar to the native lysosomal sialic acid transport system. Furthermore, we describe the effect of disease-causing mutations on the protein. We find that the majority of the mutations are associated with a complete loss of activity, while the mutations associated with the milder forms of the disease lead to reduced, but residual, function. Thus, there is a direct correlation between sialin function and the disease state. In addition, we find with one mutation that the protein is retained in the endoplasmic reticulum, indicating that altered trafficking of sialin is also associated with disease. This analysis of the molecular mechanism of sialic acid storage disorders is a further step in identifying therapeutic approaches to these diseases.

Lysozymes are intracellular acidic organelles filled with hydrolytic enzymes. They are primarily responsible for the compartmentalized degradation of macromolecules as part of an intrinsic turnover process and as a mechanism for processing endocytosed nutrients. The importance of these organelles in normal cellular functions is apparent from the phenotype of a group of degenerative diseases designated as lysosomal storage disorders (3, 4). The hallmark of these disorders is the accumulation of material within enlarged lysosomes. The typical clinical phenotype is one of a progressive multiorgan involvement with a predominance of neurological dysfunction and often early death. For many of these diseases genetic studies have identified causative mutations, most of which occur in genes encoding enzymes involved in the hydrolysis of macromolecules.

The major products of the enzymatic processes in lysosomes are small organic molecules. While the membrane-bound nature of the organelle allows for hydrolytic enzymes to be isolated from the cytosol, it also requires mechanisms for the release of these end products. Biochemical studies have identified several lysosomal transport systems that facilitate the movement of amino acids, nucleotides, lipids, vitamins, and sugars across the lysosomal membrane (5, 6). However, the molecular identification of the lysosomal transport systems has been difficult. Their intracellular localization and the presence of lysosomes or an equivalent organelle in all eukaryotic cells have hampered the use of expression cloning. Genetic studies of lysosomal storage disorders have offered some insight into the molecular mechanisms of the transport processes. For example, the gene mutated in cystinosis has been shown to encode a lysosomal cystine transporter (7), while a fatty acid transporter (NPC1) localized to lysosomes and endosomes has been shown to be the product of a gene mutated in Niemann-Pick disease Type C (8, 9). Recent evidence also indicates that CLN3, the protein mutated in one form of Batten Disease, mediates a lysosomal arginine-H⁺ antiport activity (10).

Salla disease and infantile sialic acid storage disorder (ISSD) are autosomal recessive lysosomal storage disorders with common features but differing degrees of severity (1, 2). Salla Disease is characterized by developmental delay with marked cognitive and motor impairment noticeable at 6–12 months of age. However, many affected individuals reach adulthood (11). ISSD has a more severe phenotype with intrauterine hydrops, neonatal ascites, dysmorphic features, and death by 2 years of age. The cellular pathology for both diseases consists of enlarged lysosomes filled with high concentrations of free sialic acid, an amino sugar related to neuraminic acid (12–14). The free sialic acid is derived from the oligosaccharide chains of sialylated glycoproteins and glycolipids that have been degraded in the lysosome. Previous studies have demonstrated that individuals with Salla disease and ISSD accumulate free sialic acid in their lysosomes due to a defect in a proton-coupled sialic acid transport activity (14–16). Despite clear differences in the severity of the clinical phenotypes, both

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1 The abbreviations used are: ISSD, infantile sialic acid storage disorder; EST, expressed sequence tag; HA, hemagglutinin; AP, adaptor protein; MES, 4-morpholineethanesulfonic acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; ER, endoplasmic reticulum; PDI, protein disulfide-isomerase.
forms of the disease are associated with no detectable lysosomal sialic acid transport.

Genetic studies of individuals with Salla Disease led to the determination that a gene encoding a protein designated sialin contains the causative mutations (1). Sialin is a member of the anion/cation symporter (ACS) family of proteins and has a high degree of similarity to the vesicular glutamate transporters (VGUT1–3) (17). To date 16 mutations in sialin have been associated with the disease phenotype (2). The most common mutation (R39C) is found in Salla disease. The more severe ISSD is associated with four other missense mutations (K136E, H183R, P334R, and G371V), a short deletion (Δ268–272), and 10 mutations that lead to early truncations or large deletions.

To better understand the mechanisms by which mutations may lead to disease, we sought to characterize the function of sialin. By altering a potential lysosomal targeting domain we are able to express the protein on the cell surface allowing for the use of whole cell uptake assays to measure transport activity. Using this approach we directly demonstrate that sialin is a sialic acid transporter with characteristics similar to those of the native lysosomal transport system. Through characterizing the effect of disease-associated mutations on transport activity we show a direct correlation between the degree of transport activity lost and the severity of the clinical phenotype. In addition, we demonstrate that one mutation leads to retention of the protein in the endoplasmic reticulum, suggesting that altered trafficking of sialin can also contribute to the pathophysiology of these disorders.

EXPERIMENTAL PROCEDURES

Isolation of Rat Sialin cDNA and Mutagenesis—The predicted human sialin peptide sequence was used to identify expressed sequence tags (ESTs) in the rat sequence data base (NCBI). Oligonucleotides corresponding to the 5′ and 3′ ends of the coding sequence were generated and high fidelity PCR (Turbo Pfu, Stratagene) was used to amplify the sequence from PC12 cell cDNA. The cDNA was subcloned into the expression vector pcDNA3 (Invitrogen). To introduce disease-associated mutations and restriction sites for epitope tagging, site-directed mutagenesis was carried out in parallel for the wild-type, R39C, and K136E mutants with the dileucine motifs disrupted. Transplantation was measured at 3 min as described above. For quantification of cell surface protein expression Western blots were scanned and converted to a TIFF file for analysis. Regions of interest were identified and quantified in density units using NIH Image. The corresponding region in a lane with a sample from cells transfected the wild type were subtracted from the total and subtracted from all values. Relative specific activity for each mutant was calculated from the following equation: [uptake by mutant (pmols/3 min)/uptake by wild type (pmols/3 min)]/mutant expression level (density units/wild-type expression level (density units)]

RESULTS

Mutations Associated with Disease Occur in Conserved Residues—To facilitate the characterization of the physiological role of sialin we identified a rat orthologue of the human protein. A BLAST search of available rat ESTs led to the identification of separate ESTs corresponding to the 5′ and 3′ ends of the rat sialin cDNA. Oligonucleotide primers were designed to generate a sequence corresponding to the open reading frame of the cDNA. Amplification of the sequence generated a cDNA that predicts a 495-amino acid peptide with 12 transmembrane domains and 86% identity and 90% similarity to the human protein. A similar degree of homology is also noted in comparisons of the rat or human sequences to the identified murine or ovine sequences (Fig. 1).

Sequence comparison of the mammalian proteins indicates that the disease-causing point mutations occur throughout the protein with no obvious clustering. The predicted topology suggests that the mutations occur in residues within (H183R, P334R, and G371V) or abutting (R39C, K136E) transmembrane domains. In contrast, the amino acids excised in the short deletion are in the middle of a well defined extramembrane region. The residues associated with the mutations are highly conserved among the mammalian proteins; the only variation is a single conservative substitution in the deleted segment (murine species). The absence of structure-function analysis of sialin or related proteins precludes predictions about the influence of the mutations based on their location in the protein.

Disruption of Putative Adaptor Protein-binding Domain Leads to Plasma Membrane Expression of Sialin—Measuring transport activity of recombinant sialin is complicated by the fact that the protein is likely expressed in all cells of higher eukaryotic organisms. To circumvent the problem of endogenous lysosomal sialic acid transport activity we sought to generate a form of sialin that traffics to the cell surface, thus allowing for whole cell uptake assays to measure activity of the
recombinant protein. While a plasma membrane sialic acid transport activity has been described (22), we found no measurable uptake in intact HeLa cells, suggesting that this approach would be feasible.

In analyzing sialin sequences from different species we noted a short sequence (DXXPLL) in the cytosolic amino terminus that is conserved from *Xenopus laevis* to humans (Fig. 2A). A dileucine with an acidic amino acid 4 residues upstream is a consensus motif for adaptor protein (AP) binding (23). Since interactions with AP1 or AP3 are required for proper targeting of some lysosomal membrane proteins (23), we sought to determine whether this domain in sialin is involved in trafficking.

We first replaced the amino-terminal cytosolic domain of sialin, containing the motif, with the corresponding region of NaPi-1, a structurally related protein that resides on the plasma membrane (24). When heterologously expressed in HeLa cells, the HA-tagged chimeric protein no longer displays a pattern of large puncta typical of lysosomal antigens but stains finer structures within the cell. It also appears to be present on the plasma membrane (Fig. 2B). To further refine the sequence necessary for lysosomal targeting, in separate constructs we mutated the leucines and altered the spacing between the leucines and the acidic residue. These changes have been shown to disrupt the interaction of the lysosomal protein LIMP2 with AP3 and to alter targeting of the protein (25). Replacing both leucines with alanines (L22A,L23A) or inserting two additional amino acids (alanines) between the acidic residue and the dileucine of sialin (20AAins21) resulted in an expression pattern similar to the NaPi-1-sialin chimera, indicating that this specific motif within the amino terminus facilitates lysosomal targeting of sialin (Fig. 2B).

### Sialin Is a pH-dependent Sialic Acid Transporter—Disruption of the targeting sequence enables measurements of activity at the plasma membrane through whole cell uptake assays.

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**FIG. 1. Disease-associated mutations occur in highly conserved residues.** The predicted human, rat, mouse and sheep sialin amino acid sequences are compared using the Pileup program (GCG version 10.1). Black shaded boxes indicated identity, gray boxes similarity. Missense mutations (R39C, K136E, H183R, P334R and G371V) are indicated by the individual letters above the corresponding residues in the pileup. The short deletion (Δ268–272) is indicated above the deleted amino acids. Predicted transmembrane domains (TOPPRED-GES) are indicated by the gray bars above the corresponding residues. Residues predicted to undergo N-glycosylation (N59; N71; N77; N95; N225; N302; N357), CAM kinase dependent phosphorylation (S256), Protein Kinase C dependent phosphorylation (T17; S37; T79; S269; S276) and Casein kinase II dependent phosphorylation (T72; S259; T452) are conserved to varying degrees among the mammalian orthologues.

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**Molecular Mechanisms of Free Sialic Acid Storage Disorders**
The orientation of the protein, with the luminal side facing out, is such that whole cell uptake corresponds to the transport of sialic acid out of the lysosome. Transporters are often reversible, but asymmetries in relative affinity can exist at the two sides of the transporter. Even though the physiologically relevant activity for the lysosomal sialic acid transport system is to mediate the outward movement of sialic acid, the most comprehensive studies on the native transport system have relied on the reversibility of the transport system and measured uptake into intact lysosomes (26–28). With sialin mistargeted to the cell surface, however, the activity measured will more directly reflect the physiologically relevant function of the protein.

For initial studies, to make the extracellular environment approximate the acidic lysosomal lumen, the cells were incubated in a Krebs-Ringer solution buffered with MES at pH 5.5. A marked time-dependent increase in the uptake of sialic acid is seen in cells expressing plasma membrane targeted sialin compared with cells expressing another lysosomal transporter, LYAAT1 (Fig. 3, A and B). The activity is saturable with a \( K_m \) of ~0.8 mM, which is similar to, but slightly higher than, the value reported for the uptake of sialic acid into lysosomes (Fig. 3C). Sialic acid uptake is not affected by switching to a Na\(^+\) - and Cl\(^-\)-free MES-buffered sucrose solution (data not shown), indicating that the activity is not dependent on, or inhibited by, either of these ions. However, uptake is reduced by two known inhibitors of lysosomal sialic acid uptake, glucuronic acid and DIDS (Fig. 3D).

A large pH gradient exists across the lysosomal membrane and several lysosomal transport systems, including the sialic acid transport system, are coupled to this gradient. To determine the role of pH in the activity of the recombinant protein, both the pH of the buffer solution and the pH gradient across the plasma membrane were varied. Increasing the buffer pH from 5.5 to 6.5 markedly decreases sialin mediated transport, and essentially no transport is detected when the extracellular solution is buffered at approximately the same pH as the cytoplasm (pH 7.5) (Fig. 3E). In addition, uptake is reduced by ~80% at pH 5.5 by abolishing the pH gradient with nigericin, an ionophore that mediates H\(^+\)/K\(^+\) exchange (Fig. 3F). On the other hand, transport activity is not affected by specific dissipation of the electrical gradient using the K\(^+\) ionophore valinomycin, suggesting that transport is electroneutral.

A Subset of Mutations Is Associated with Residual Transport Activity—After confirming that sialin is a sialic acid transporter, we next sought to determine whether mutations associated with disease alter the intrinsic transport activity of the protein. To do this, we disrupted the putative AP-binding domain of proteins containing the disease-associated mutations (R39C, K136E, H183R, P334R, G371V, and \( \Delta \)268–272(SSLRN)). This leads to plasma membrane expression of all of the proteins with the exception of G371V, as indicated by the pattern of immunofluorescence staining (Fig. 4A) and cell surface biotinylation (data not shown). Measuring total uptake at 5 min (Fig. 4B) demonstrates that the transport activity of the R39C and K136E mutants is reduced. Whereas, essentially no transport activity can be detected for the other mutants (H183R, P334R, G371V, and \( \Delta \)268–272(SSLRN)). The absence of measurable activity associated with the H183R and P334R mutations and the short deletion despite plasma membrane expression strongly suggests that these mutations abolish transporter activity. However, since G371V is not present at detectable levels on the plasma membrane, with this approach we cannot determine whether the mutation influences the intrinsic transport activity of the protein.

Although a partial reduction in transport activity could lead to disease, the absence of a phenotype in heterozygote carriers of the free sialic acid storage disorders, who have approximately half the normal lysosomal sialic acid transport activity (27), suggests that activity would need to be reduced by more than 50%. Therefore, we wanted to know, more precisely, the effect of the R39C and K136E mutations on activity. To accomplish this, we analyzed sialic acid uptake relative to the amount of biotinylated cell surface protein expression. For both mutants, uptake and plasma membrane expression were determined relative to transfected wild-type protein. Normal-
The G371V Mutation Leads to Retention of Sialin in the ER—In a previous report in which the localization of sialin was compared with LAMP1, a lysosomal late endosomal marker, recombinant proteins with the Salla mutation and the short deletion were found to be mislocalized, leading the authors to suggest that improper targeting could contribute to the defect associated with these mutations (29). In trying to determine the extent to which other mutations influence trafficking of sialin we found that wild-type sialin only partially localizes with LAMP1 and LAMP2 in HeLa cells (data not shown), suggesting that colocalization studies with these endogenous antigens would be difficult. Therefore, to determine whether mutations affect trafficking we compared wild-type and mutant proteins expressed in the same cell using differential epitope tagging.

As expected, simultaneous expression of HA-tagged and Myc-tagged wild-type sialin leads to colocalization of the two epitopes by immunofluorescence staining (Fig. 5A). Analysis of
cells cotransfected with Myc-tagged wild-type sialin and HA-tagged R39C mutant demonstrates a predominant staining pattern of complete overlap with only rare cells with partial overlap (Fig. 5A). This colocalization is independent of expression levels or time after transfection (data not shown). Similar colocalization with the wild-type protein is seen with other mutants including K136E, H183R, P334R, and the short deletion (Fig. 5A).

A striking differential localization is only seen for G371V. This mutant colocalizes with PDI, an endoplasmic reticulum protein, suggesting retention in this compartment (Fig. 5B).

Interestingly the combination of G371V with the disrupted putative AP-binding domain also colocalizes with PDI (data not shown), consistent with sialin interacting with an adaptor protein in a post-ER compartment.

**DISCUSSION**

Characterization of the biochemical phenotype of Salla disease and infantile sialic acid storage disorder led to the identification of a lysosomal sialic acid transport activity (15, 26), and subsequent genetic studies suggested that sialin, the protein mutated in these diseases, mediates this activity (1). Here we present data supporting a direct role for sialin in sialic acid transport and describe the effects of disease-associated mutations on the transport activity and trafficking of the protein.

The analysis of the sialin sequence from different species highlights two interesting points. First, the missense mutations associated with Salla disease and ISSD occur in absolutely conserved residues within or abutting transmembrane domains. This degree of conservation is consistent with these residues playing a crucial role in the function of the protein. Furthermore, the association of these residues with membranous segments of the protein fits with a role in recognition or translocation of the substrate or in the proper folding of the protein within the membrane.

The second point derived from a comparison of the sequences is the presence of a conserved putative AP-binding domain in the amino terminus of sialin. The motif, an acidic amino acid four residues upstream of a dileucine, is similar to domains required for interactions between adaptor proteins and their binding partners, including the interaction between AP3 and the lysosomal protein LIMP2 (23, 25). Replacing the entire amino terminus, mutating the dileucine residues, or inserting two additional amino acids between the dileucine and the requisite upstream acidic residue all increase plasma membrane expression of sialin. In addition, a phosphorylatable residue precedes the consensus motif in all of the mammalian sialin sequences. Interestingly a serine precedes similar dileucine motifs in the CD3-γ chain of the T-cell receptor (SDKQTLL)
and the CD4 coreceptor (SQIKRLL) (30–32). Phosphorylation of these serines induces a rapid internalization of the proteins and targeting to lysosomes where the proteins are degraded. If localization of sialin is controlled in a manner parallel to these highly regulated components of receptors, it suggests that the function of sialin may be more complex than simply mediating the efflux of a degradative product from lysosomes.

The plasma membrane localization associated with the al-

**FIG. 5. G371V mutation is associated with retention in the endoplasmic reticulum.** A, HA-tagged proteins with disease-associated mutations (*middle column*) were coexpressed with Myc-tagged wild-type sialin (*left column*) in HeLa cells. Overlay of confocal images (*right column*) demonstrates that proteins with the R39C, K136E, H183R, and P334R mutations, as well as the short deletion, colocalize with the wild-type protein. B, a marked difference in staining patterns is demonstrated for G371V. Double labeling studies with PDI, an endoplasmic reticulum marker, suggests that G371V is retained in the ER. The scale bars indicate 10 μM.
tered amino terminus enabled us to measure transport, not in lysosomes, but at the cell surface, thus circumventing the background activity associated with endogenously expressed sialin. The transport activity of the recombinant sialin retains the essential characteristics of the previously described native lysosomal sialic acid transport activity (26, 28), confirming the predicted role for the protein. The transport activity is inhibited by glucuronic acid, an acidic sugar that is a competitive inhibitor of the lysosomal sialic acid transport. Although it occurs less commonly in glycoproteins and glycolipids, glucuronic acid is also a physiological substrate for the transporter and accumulates in the lysosomes of individuals with the free sialic acid storage disorders (26, 33). The stilbene derivative DIDS, a anion channel blocker, inhibits VGLUT1 transport function (34) as well as sialin-mediated transport.

Lysosomal transport systems can mediate facilitated diffusion or can be coupled to the proton electrochemical gradient through proton cotransport. Our data and previous data on the lysosomal transport system (26) suggest that sialin is directly coupled to the proton gradient and that there is a 1:1 stoichiometry between the sugar and protons. However, whether or not sialin plays a significant role in regulating lysosomal pH, either through coupled movement of protons (as has been suggested for the lysosomal arginine transporter CLN3 (35, 36)) or through an anion conductance (as has been described for the structurally related NaPi-1 and VGLUT1 (24, 34, 37)), remains to be determined.

For sialin to carry out its predicted function it must be localized to lysosomes, have an appropriate affinity for sialic acid, and couple sialic acid movement to the cotransport of protons. Therefore, mutations that lead to disease can mediate their effects through a variety of mechanisms. While large deletions and truncations are likely to lead to a marked reduction or absence of the protein, point mutations may lead to altered transport activity or influence stability and/or trafficking of the protein. In analyzing the effect of the disease mutations on transport function we found that the Salla (R39C) and K136E mutants retain low levels of intrinsic activity. Whereas, for H183R, P334R, and Δ268–272(SSLRN) no activity could be detected despite plasma membrane expression of the proteins. G371V appears to be retained in the ER so it is impossible to determine its activity by plasma membrane uptake assays.

For the mutants with no transport activity, the molecular mechanisms leading to loss of function remain to be determined. Some possibilities are suggested by the residues in which the mutations occur. For example, histidine has a pKa of ~6.0, suggesting that within a physiologically relevant pH range His183, which lies within a predicted transmembrane domain, could accept and transfer protons. Indeed, in other proton-coupled transporters histidine residues within membrane segments have been implicated in proton transfer (38, 39). Interestingly, the corresponding mutation in the related VGLUT1 retains activity.2 Although VGLUT1-mediated transport is driven by a proton electrochemical gradient, it is dependent on the electrical, not the chemical component, suggesting that its transport activity does not involve proton movement (34). Thus, His183 may be directly involved in proton coupling, a mechanism necessary for sialin function but not VGLUT1 function.

As an underlying mechanism by which mutations lead to a loss of function, residue changes may alter the structure or stability of the proteins. For instance, the short deletion may interfere with proper folding of the protein by limiting the flexibility between the predicted transmembrane domains that the loop appears to bridge. In addition, both proline and glycine residues are important in transmembrane protein structure; prolines are helix-breaking residues often occurring at the ends of transmembrane segments, and glycines can allow for appropriate packing of transmembrane domains (40). This suggests that the effect of the P334R and G371V mutations may be on the tertiary structure of sialin. Interestingly, ER retention, as seen with the G371V mutation, is common to disease-causing mutations in two other polytopic proteins, CFTR and aquaporin-2 (41–43). Recently, in an animal model with mutant CFTR, inhibition of an ER-associated degradation has been shown to increase expression levels and ameliorate the phenotype of the disease (44, 45). Whether or not a similar effect occurs for the G371V mutation in sialin remains to be determined.

Perhaps most interesting are the results of our analysis of the Salla disease mutation (R39C) and the K136E mutation. In each case, residual activity is clearly present, and each protein appears to colocalize with the wild-type protein, suggesting proper trafficking. It is important to note that both mutations are associated with milder forms of the disease (2). The K136E mutation has only been reported in a single case, in a compound heterozygous individual with the R39C mutation where the clinical phenotype was intermediate between Salla disease and ISSD. Compared with ISSD, Salla disease is clinically less severe and is associated with lower levels of free sialic acid in both urine and cells cultured from patients. An unresolved issue has been the difference in phenotypes for Salla disease and ISSD, despite a complete absence of measurable sialic acid transport activity in lysosomes derived from patients with both diseases (15, 16, 27). Although this has been attributed to a lack of sensitivity in the methods used to measure lysosomal transport activity, some uncertainty has persisted. Our data demonstrating that the Salla mutation retains residual activity reconcile this issue and further suggest that there is a direct correlation between clinical phenotype and transporter function.

In characterizing the function of sialin, we have advanced our understanding of the mechanisms that underlie Salla disease and ISSD. We have demonstrated both a direct role for the protein in the transmembrane transport of sialic acid and a loss or severe reduction in transport activity in several different disease-associated mutations. The loss of intrinsic function suggests crucial roles in the transport process for the mutated residues, and further comparison of the effect of similar mutations in the related vesicular glutamate transporters may reveal important mechanistic differences among these structurally related, but functionally divergent, proteins. In addition, the retained activity associated with specific mutations suggests that therapeutic approaches directed at increasing the expression or stability of sialin may prove beneficial in these cases.

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