# Lysosomal membrane transport proteins and their significance in human genetic disease

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Front cover: My interest in transport is not restricted to my work, but is extended into my private life. Lay-out: Ridderprint, C. Poot.

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# Lysosomal membrane transport proteins and their significance in human genetic disease

Lysosomale membraan transport eiwitten en hun rol in erfelijke ziekten bij de mens

**Proefschrift**

Ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

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**Adriana Catharina Havelaar**

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*Voor mijn ouders en Jan Willem*

## CONTENTS

### Chapter 1 GENERAL INTRODUCTION



### Chapter 2 DISCUSSION OF THE EXPERIMENTAL WORK



### Chapter 3 PUBLICATIONS

# Chapter 1 GENERAL INTRODUCTION

## 1.1 Rationale and aims of the study

Lysosomes are intracellular acidic organelles, which are mainly responsible for the degradation of a variety of intra- and extracellular macromolecules. The lysosomal membrane contains transport proteins for both export and import. A few importers are known: these are responsible for the uptake of a variety of small molecules or ions, suggesting a role for the lysosome in the regulation of certain metabolic processes. Many exporters have been characterized: these allow the small degradation products to leave the lysosome to be either reutilized or excreted by the cell. Exporters have been described for a variety of small molecules including amino acids, sugars and ions. Two human genetic disorders are due to defective export systems, cystinosis and sialic acid storage disease.

The efflux of free sialic acid is impaired in the clinically heterogeneous group of sialic acid storage disease (SASD)(Mancini et al., 1986; Renlund et al., 1986; Tietze et al., 1989). This autosomal recessive inherited disorder is characterized by lysosomal accumulation of free sialic acid and excessive sialuria. A proton gradient-driven transporter with an essential metabolic function in the disposal of the acid sugars, sialic acid and glucuronic acid, is the primary genetic defect in Salla disease and infantile sialic acid storage disease, the two clinical variants of SASD (Mancini et al., 1989, 1991).

One aim of this study was to elucidate the molecular structure and functional properties of the lysosomal sialic acid transporter and to understand the molecular defect(s) in the clinical heterogeneous forms of SASD. Knowledge about the molecular structure of a lysosomal transporter will eventually advance our understanding of the biogenesis, regulation and targeting of these transporters in general. At the start of this study in 1993, none of the lysosomal transport proteins was purified nor were their encoding genes cloned. We have purified the lysosomal sialic acid transport protein and characterized its functional properties.

Another objective of our study was to investigate a possible role for lysosomes and their membrane transporters in the regulation of the intracellular metabolism of heavy metal ions, also in relation to human genetic diseases. A possible route of copper excretion is via exocytosis of lysosomal contents into biliary canaliculi (Gross et al., 1989). So far, direct evidence that lysosomes are able to take up or excrete, sequester and mobilize heavy metal ions by specific transporters has been lacking. We demonstrated the presence of a novel heavy metal ion transporter in the lysosomal membrane. Its possible role in a copper accumulation disorder, Wilson disease, was investigated by transport assays in an existing animal model for this disease.

## 1.2 Lysosomal membrane transport proteins

Initially, it was thought that small molecules could cross the lysosomal membrane via simple diffusion (Lloyd, 1969 and 1971; Reijngoud and Tager, 1977). This view was largely disproved after the demonstration of substrate-specific transport proteins. In particular, the existence of two inherited lysosomal storage diseases due to defective export mechanisms (cystinosis and Salla disease) has contributed to the understanding of transport across the lysosomal membrane. Since the discovery of a lysosomal cystine carrier, defective in cystinosis (Gahl et al., 1982), more than 20 specific transport proteins have been characterized in the lysosomal membrane. Most of them function as exporters and only a few as importers. The lysosomal transporters have a specificity for amino acids, sugars, nucleosides, inorganic ions, and vitamins (Fig. 1). Until 1998, all knowledge about lysosomal transport proteins was based on the biochemical (kinetic) characteristics of transport.

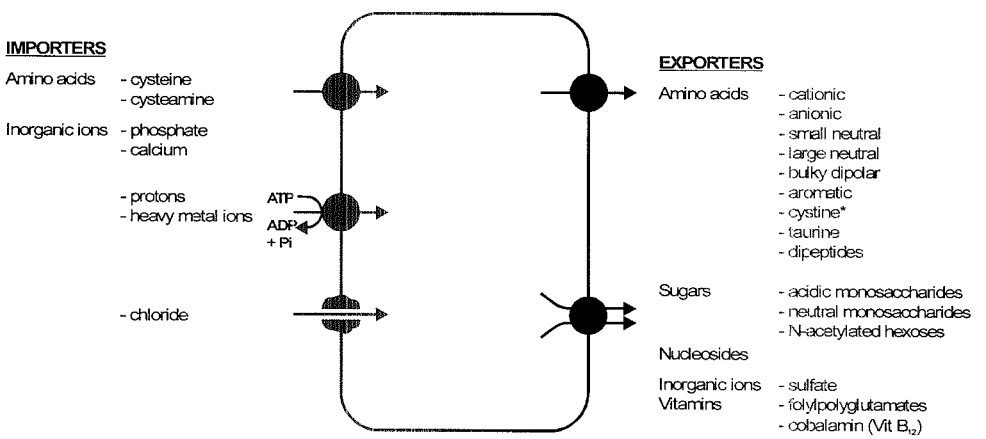


**Figure 1.** Schematic diagram of a lysosome showing the characterized lysosomal membrane transport systems. *Defective transport system in cystinosis (cystine transporter) or in sialic acid storage disease (acidic monosaccharide transporter).

In Table 1 the different lysosomal transport systems are listed according to their substrate specificity, and their most important features are summarized. For detailed information regarding each system, readers are referred to the references indicated in Table 1 and previous reviews (Mancini, 1991; Pisoni and Thoene, 1991; Chou et al., 1992). The molecular and functional properties of the better characterized lysosomal transport systems are discussed below.

**Table 1. Lysosomal membrane transport systems classified according to their substrate specificity.**

| System | Substrate | Potential substrates | Transport type* | Source | Reference |
|---|---|---|---|---|---|
| **Amino acids** | | | | | |
| (c)** cationic | L-lys, arg | L-orn, cysteamine-cysteine disulfide | secondary active ? | fibroblasts | Pisoni et al., '85,'87a |
| (d) anionic | L-glu, asp | L-α-aminoadipate | passive | fibroblasts | Collarini et al., '89 |
| (e) small neutral | L-ala, ser, thr | L-leu | passive | fibroblasts | Pisoni et al., '87b |
| (f) small neutral | L-pro (major route), ala | β-amino acids (< 3 carbons long) sarcosine, N-methyl-L-ala | passive | fibroblasts | Pisoni et al., '87b |
| (p) small neutral | L-pro (minor route), 3,4-dehydro-L-proline | - | passive | fibroblasts | Pisoni et al., '87b |
| (h) large neutral | L-tyr, leu, ileu, trp, phe, his, val, met | D-try, monoiodotyrosine | passive | rat thyroid cells | Bernar et al., '86 Andersson et al., '90 |
| (l) bulky dipolar | L-leu, val | Norleu | passive | fibroblasts | Stewart et al., '89 |
| (t) aromatic | L-trp, phe | D-trp | passive | fibroblasts | Stewart et al., '89 |
| cystine | L-cystine, selenocystine | cystathionine, cysteamine-cysteine disulfide, cystamine | passive? | leucocytes rat liver | Gahl et al., '82 Jonas et al., '82 |
| cysteine | L-cysteine | - | secondary active ? | fibroblasts | Pisoni et al., '90, Pisoni and Velilla, '95 |
| cysteamine | cysteamine | aminothiols, aminosulfides, thiocholine | secondary active ? | fibroblasts | Pisoni et al., '95 |
| taurine | taurine | β-ala, hypotaurine | secondary active ? | rat liver | Vadgama et al. '91 |
| dipeptides | gly-gln | dipeptides, tripeptides | secondary active ? | rat liver | Thamotharan et al., '97 |
| **Sugars** | | | | | |
| acidic sugars | Neu5Ac, GlcA | IdoA, L-lactate, α-ketoglutarate | secondary active | rat liver, fibroblasts lymphoblasts | Mancini et al., '89, '91 |
| neutral monohexoses | D-glucose | D-galactose, D-mannose, D- and L-fucose | passive | rat liver, fibroblasts rat liver | Manicini et al., '90,'91 Jonas et al., '90 |

| System | Substrate | Potential substrates | Transport type* | Source | Reference |
|---|---|---|---|---|---|
| N-acetylated hexoses | N-acetylglucosamine N-acetyl-galactosamine | - | passive | rat liver | Jonas et al., '89 |
| **Nucleosides** | purines, pyrimidines | - | passive | fibroblasts | Pisoni and Thoene, '89 |
| **Inorganic ions** | | | | | |
| $H^+$ pump/ATPase | protons | - | primary active | rat liver | Schneider, '81 |
| chloride channel | $Cl^-$ | - | channel | rat liver | Tilly et al., '92 |
| phosphate ion | $HPO_4^{2-}$ | ascorbate | passive | fibroblasts | Pisoni, '91 |
| sulfate ion | $SO_4^{2-}$ | molybdate | secondary active? | rat liver | Jonas and Jobe, '90 |
| calcium ion | $Ca^{2+}$ | $Cd^{2+}$, $Hg^{2+}$, $Zn^{2+}$,$Mg^{2+}$ | passive | fibroblasts | Lemons and Thoene, '91 |
| heavy metal ions | $Ag^+$ | $Cu^{2+}$, $Cd^{2+}$ | primary active | rat liver | Publication IV |
| **Vitamins** | | | | | |
| folylpolyglutamates | | methotrexate polyglutamates | passive | S180 cells | Barrueco and Sirotnak, '91 |
| cobalamin (Vit.$B_{12}$) | cyanocobalamin | adenosylcobalamin, methylcobalamin | secondary active? | rat liver | Idriss and Jonas, '91 |
| **Unknown mechanism** | | | | | |
| cholesterol | | | | | |
| iron ion | | | | | |

*For a description of the different transport mechanisms see chapter 1.3
** (c)-(t) correspond to the nomenclature for the lysosomal amino acid transport systems.

## Amino acid transport

The inherited lysosomal storage disease cystinosis results from impaired transport of **cystine**, a disulfide amino acid, out of lysosomes (Gahl et al., 1982; Jonas et al., 1982). The biochemical characteristics of the lysosomal cystine transport system have been investigated in a variety of cell types (Gahl et al., 1983; Pisoni et al., 1985, Greene et al., 1990). Besides L-cystine it also recognizes cystathionine, cysteamine-cysteine disulfide and cystamine. The system is sensitive to changes of the membrane potential and to pH. However, transport of cystine is not coupled to transport of other ions (secondary active transport) (Smith et al., 1987).

The cystinosis gene has recently been identified by positional cloning (Town et al., 1998). The gene, CTNS, encodes cystinosin, a 367 amino acid protein with six or seven predicted transmembrane domains. Most established solute transport proteins possess six or twelve transmembrane domains (see Chapter 1.3). The protein appears to have an uncleavable N-terminal signal sequence. An indication for lysosomal targeting of cystinosin is the presence of the GYXX-hydrophobic amino acid motif close to the C-terminus, a feature common to several lysosomal membrane proteins with unknown function (Hunziker and Geuze, 1996). Besides this hydrophobic sequence, also a positively-charged sequence is present near the C terminus of cystinosin, indicating similarities with the lysosome-associated membrane glycoproteins (LAMPs). Another similarity between these proteins is the presence of many potential N-glycosylation sites at the N-terminus. These similarities with lysosomal membrane proteins indicate that cystinosin is likely to be a lysosomal membrane protein (Town et al., 1998). Elucidation of the complete topology of cystinosin as well as its functional characteristics will provide the answers on many questions concerning the function and regulation of lysosomal cystine transport.

**Cysteamine** is therapeutically important in the treatment of cystinosis as it is able to enter lysosomes, and to react with free cystine to form free cysteine and the mixed disulfide of cysteamine and cysteine (Thoene et al., 1976). The latter compound, an analog of lysine, exits lysosomes via the lysosomal membrane carrier for cationic amino acids (system c), which is intact in cystinosis (Pisoni et al., 1985). After the characterization of the cysteamine transporter it became clear how cysteamine enters the lysosome (Pisoni et al., 1995). The lysosomal uptake of cysteamine showed *in vitro* a dramatic response to pH, with a 50-fold higher rate of uptake at pH 8.2 than at pH 5, indicating an import rather than an export function. The substrate specificity of this carrier is highly unusual among known transport systems in that all analogs recognized are either aminothiols or aminosulfides, and contain a sulfur atom and amino group separated by 2 carbon atoms. The same research group had previously characterized a **cysteine**-specific lysosomal transport system (Pisoni et al., 1990). Only cysteamine, which is the decarboxylated analog of cysteine, could strongly inhibit lysosomal [$^{35}$S]cysteine uptake.

At that time, its translocation by the cysteine carrier could not be demonstrated, because of the unavailability of radiolabeled cysteamine. Now, it can be deduced that cysteamine is transported into lysosomes by its own specific transport system. Like the cysteamine transporter, the lysosomal cysteine transporter also appears to function as an importer rather than an exporter (Pisoni et al., 1990). The cysteine-specific import route may play an important role in supporting lysosomal proteolysis by providing thiol for the lysosomal thiol-dependent proteases and by reducing protein disulfide bridges. In this way proteins are allowed to unfold, facilitating their degradation. The role of this transport system in delivering cysteine into lysosomes is supported by the observation that the activity of the transporter increases 7-10-fold between pH 6 and pH 7.3, to be maximally active in the neutral pH range.

Another transport system that plays a role in lysosomal proteolysis is a **dipeptide** transporter (Bird and Lloyd, 1990). In a recent study, transport of radiolabeled Gly-Gln dipeptide was demonstrated into rat liver lysosomal membrane vesicles by a single transporter with a substrate specificity for di- and tripeptides (Thamotharan et al., 1997). The transporter is stimulated by an acidic pH and a membrane potential and it showed a 1:1 stoichiometry between Gly-Gln and $H^+$. Since the degradation of dipeptides takes place in the cytosol (Bouma et al., 1976), this transporter provides an active mechanism for completion of protein degradation.

### Sugar transport

Previous investigations have demonstrated the existence of three lysosomal carbohydrate transport systems. One of the lysosomal sugar transport proteins is a carrier specific for **acidic monosaccharides** like **sialic acid and glucuronic acid**, degradation products of glycoproteins, glycolipids or glycosaminoglycans. Its existence and properties were clarified *in vitro* using lysosomal membrane vesicles, a technique used for reliable kinetic characterization of lysosomal transport systems. Transport of these acidic monosaccharides across the lysosomal membrane is a carrier-mediated process, driven by a proton-gradient (Mancini et al., 1989). Subsequent studies on the sialic acid transporter in lysosomal membranes from human fibroblasts demonstrated that the $H^+$-driven transport of both sialic acid and glucuronic acid is deficient in patients with the different clinical forms of sialic acid storage disease (SASD). Evidence that the transport defect represents the primary genetic mutation came from the observation of intermediate transport rates in obligate heterozygotes for this autosomal recessive disease (Mancini et al., 1991). We also developed a functional reconstitution system for the sialic acid transporter into proteoliposomes (Mancini et al., 1992a). This system provided the tool to start the purification of the lysosomal sialic acid transporter from rat liver lysosomal membranes (Publication I). Functional characterization studies showed that the lysosomal sialic acid carrier transports besides structurally different acidic monosaccharides, also

other non-sugar mono- and dicarboxylated anions, like L-lactate and α-ketoglutarate (Publication I and II). Apparently, this protein shows functional similarities with certain anion transporters of molecular classified transporter families (Publication II). Details about the purification protocol and the functional properties of the lysosomal sialic acid transporter will be discussed in Chapter 2.

A second lysosomal sugar transport system is a **glucose** transporter for the translocation of neutral hexoses like D-glucose, D-mannose, D-galactose and D- and L-fucose (Mancini et al., 1990; Jonas et al., 1990a). The acidic pH optimum of this transporter discriminates it from other glucose carriers (Olsen and Pessin, 1996).

A third lysosomal sugar transport system mediates the specific translocation of the two acetylated amino sugars **N-acetyl-D-glucosamine and N-acetyl-D-galactosamine**, which are also degradation products of glycoproteins, glycosphingolipids, and glycosaminoglycans (Jonas et al., 1989).

### Inorganic ion transport

The most prominent of the lysosomal ion transporters is the **$Mg^{2+}$-ATP-dependent electrogenic proton pump ($H^+$-ATPase)**. This pump is involved in the maintenance of the acidic pH within lysosomes (Dell'Antone, 1979; Schneider, 1981; Ohkuma et al., 1982). Similar proton pumps have been identified on a variety of membranes of acidic organelles of vacuolar systems as well as on the plasma membranes of some cells, and are classified as vacuolar-type $H^+$-ATPases (V-type ATPases)(Finbow and Harrison, 1997). Recently, the lysosomal $H^+$-ATPase has been purified from rat liver, and shown to have the typical subunit structure of vacuolar $H^+$-ATPases of 7 or 8 subunits (Arai et al., 1993). Anions like chloride dissipate the electrochemical gradient of protons across the vacuolar membrane created by electrogenic proton transport. Therefore, a V-type $H^+$-ATPase and an anion channel (*e.g.* chloride) or a transporter (*e.g.* sulfate) often coexist in endomembrane compartments (Mellman et al., 1986). Such a **chloride-specific anion channel** has also been described in the lysosomal membrane (Tilly et al., 1992). Further studies are required to clarify the role of ions and their channels in the maintenance of intralysosomal pH. The vacuolar proton pump not only acidifies the vesicle interior, but also provides an energy source for driving a variety of coupled transporters like the sialic acid transporter (Mancini et al., 1989;van Dyke,1996).

Another lysosomal transporter for inorganic ions is the **phosphate** transporter. It has been shown in fibroblast lysosomes that phosphate, released upon the ATP hydrolysis by the proton pump, is taken up by the lysosome by a specific transport system (Pisoni, 1991). Rapid metabolism of phosphate in the lysosome to trichloroacetic acid-soluble and -insoluble products prevents the measurements of efflux of phosphate *in vitro*. However, the efflux of phosphate can occur, since time-dependent uptake curves reach steady-state levels indicating equilibrium between influx and efflux. It is still unclear whether the

phosphate produced by intralysosomal hydrolysis of nucleic acids (cytoplasmic RNA) is leaving the lysosome via this transporter or is further metabolized. The **nucleosides** are leaving the lysosomes via a specific transport system (Pisoni and Thoene, 1989). The possibility remains that another phosphate carrier serves mainly for the export of phosphate. To study this more precisely, lysosomal membrane vesicles are required in subsequent studies, in stead of intact lysosomes.

**Sulfate** produced by the degradation of glycosaminoglycans and sulfolipids exits the lysosomes by a specific carrier-mediated system (Jonas and Jobe, 1990b). This anion transporter is specific for sulfate and possibly for molybdate. Under physiological conditions this transport system may also be subject to regulation by thyroid hormone (Chou et al., 1994). It has some structural and functional similarities with the Band 3 anion exchanger (Koetters et al., 1995). This erythrocyte specific anion transporter transports L-lactate and a wide range of other aliphatic monocarboxylates. Both systems have common sensitivities to a variety of inhibitors, some of which modify either lysine or arginine residues in their substrate binding site. Unique for the lysosomal sulfate transport system is the inhibitory effect of cupric ions, known to interact with histidine and thiol groups (Koetters et al., 1995a). This information could be helpful in the elucidation of the structure and function of the sulfate transporter. Recent studies with thiol blocking agents revealed that lysosomal sulfate transport is dependent on sulfhydryl groups (Chou et al., 1998). In order to get more insight into the structure and function of this transporter isolation of the protein and gene are required. A method for reconstitution of sulfate transport into artificial membrane vesicles has been described (Koetters et al., 1995b). Sulfhydryl affinity chromatography might be a suitable method for protein purification (Chou et al., 1998).

### Inorganic ion homeostasis

So far, all characterized lysosomal transport systems are involved in either the regulation of the osmotic balance between lysosomes and the cytosol, or the export or import of small molecules. Lysosomes and their membrane transporters also appear to participate in the regulation of the intracellular homeostasis of certain inorganic ions.

Maintenance of a constant low cytosolic $Ca^{2+}$ is essential for normal cellular function. Lemons and Thoene (1991) demonstrated the uptake of considerable amounts of $Ca^{2+}$ by human fibroblast lysosomes. Recently, a possible role for lysosomes in intracellular $Ca^{2+}$ signaling has been investigated in MDCK cells (Haller et al., 1996). Results of this study suggested that $Ca^{2+}$ accumulates in lysosomal vesicles, which appeared to be functionally coupled to inositol triphosphate ($InsP_3$) sensitive $Ca^{2+}$ stores (Gosh et al., 1989) and may therefore play a role in intracellular $Ca^{2+}$ signaling.

For many years, lysosomes have been known to play a role in the regulation of the intracellular homeostasis of heavy metal ions. Heavy metal ions like copper and iron are

required in trace amounts for the normal functioning of many biological processes, but are toxic in excess due to their redox properties. Therefore, intracellular heavy metal ions are bound tightly to specific proteins, like metallothionein for copper and ferritin for iron. The involvement of lysosomal degradation of ferritin in iron recycling has been shown previously (Ringeling et al., 1989; Radisky and Kaplan, 1998). The mechanism by which the lysosomal iron is transported into the cytosol is yet unclear. Accumulation of iron-protein complexes in hepatocyte lysosomes has been observed in hereditary hemochromatosis, one of the most common recessive inherited disorders (Stal et al., 1990)

The detrimentous effects of copper imbalance are demonstrated in two known genetic disorders in humans, Menkes disease and Wilson disease. Both diseases are caused by genetic defects in distinct steps of copper metabolism (DiDonato and Sarkar, 1997). The basic defect in Wilson disease must be related to the inability to incorporate copper into ceruloplasmin and to excrete copper into the bile. It is known that in the case of hepatic copper overload the major route of copper excretion is via exocytosis of lysosomal contents into biliary canaliculi (Gross et al., 1989). So far, nothing was known about the lysosomal mechanism(s) to take up or export, sequester and mobilize heavy metal ions.

We decided to characterize heavy metal ion transport through the lysosomal membrane, using highly purified lysosomal membrane vesicles from rat liver. As shown by the development of a more suitable diagnostic test for Menkes disease (Publication III), radioactive copper, which is not commonly available and has a very short physical half life, can be replaced by radioactive silver in copper transport studies. Radioactive silver is commercially available and has a longer more convenient physical half life. These studies allowed the identification of a lysosomal heavy metal transporter (P-type ATPase) with a specificity for silver, copper and cadmium ions (Publication IV). The biochemical characteristics of this transporter and its potential relevance to Wilson disease are discussed in Chapter 2.

## 1.3 Molecular characteristics of membrane transport proteins

Until 1998, the molecular structures of lysosomal membrane transport proteins were unknown, since none of them had been purified or cloned. More knowledge is available about prokaryotic transporters and many eukaryotic transporters present in other cellular membranes, like the plasma membrane or the mitochondrial membranes.

### Intracellular localization

Membrane transport proteins are of vital importance for the maintenance of cellular homeostasis. They are responsible for the uptake of essential nutrients, excretion of metabolic waste products, and the regulation of intracellular ion concentrations. They occur in all types of biological membranes, as illustrated in Fig. 2. Most well-characterized transport proteins are ubiquitously present in the plasma membrane of all cells (e.g. $Na^+/ K^+$ pump). Others are sited in plasma membranes of specific cells (like the acetylcholine receptor at the plasma membrane of skeletal muscle cells) or in membranes of specific intracellular organelles (like the cystine transporter and the sialic acid transporter in the lysosomal membrane).

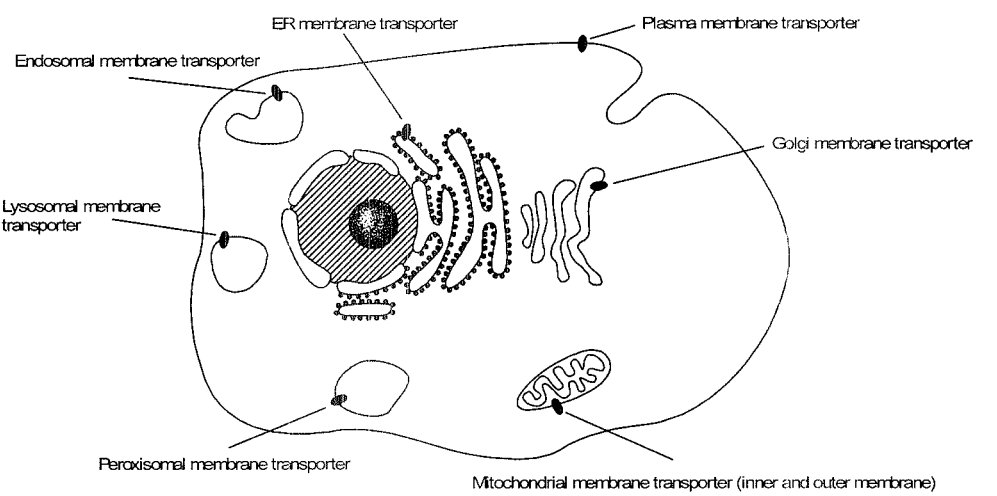


**Figure 2.** Intracellular localization of transport proteins at different membranes.

Which molecular features of the transporter proteins determine their cellular localization? Specific targeting signals are known for matrix proteins and single-spanning membrane proteins with different intracellular localizations, including the endoplasmatic reticulum, mitochondria, lysosomes and peroxisomes. The targeting signals for multispanning membrane proteins, like transport proteins, are less characterized, but

become more and more known with the increasing availability of the primary sequences of membrane transport proteins. Mitochondrial and peroxisomal membrane proteins do not appear to have the same targeting signals as matrix proteins of these organelles. A putative peroxisomal membrane targeting signal has been identified in a protein of *Candida boidinii* (PMP47), which has six transmembrane domains and is homologous to a family of mitochondrial carriers. A stretch of basic amino acids in the intervening loop between transmembrane domains 4 and 5 is responsible for the sorting of PMP47to the peroxisomal membrane (Dyer et al., 1996). Mitochondrial membrane transport proteins share a tripartite primary structure, made up of related sequences about 100 amino acids in length. Each repetitive element contains two hydrophobic stretches separated by an extensive hydrophilic region (Palmieri, 1994). Import of these proteins into the mitochondrial membrane occurs via specific TOM (outer membrane translocase) and TIM (inner membrane translocase) systems (Koehler et al., 1998). The putative zinc-fingers of TIM proteins probably recognize one or more internal targeting signals in the mitochondrial membrane transport protein (Sirrenberg et al., 1998). However, these internal targeting signals have not yet been identified.

The importance of transmembrane domains in intracellular targeting has been demonstrated for a copper P-type ATPase (ATP7A), which is mutated in patients with the human X-linked recessive disorder of copper metabolism, Menkes disease (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). ATP7A is located at the *trans*-Golgi network and is predicted to supply copper to copper-dependent enzymes (Petris et al., 1996). However, under conditions of elevated extracellular copper, the ATP7A protein undergoes a rapid relocalization to the plasma membrane where it functions in the efflux of copper from the cell (Petris et al., 1996). Recycling between the *trans*-Golgi network and the plasma membrane has also been shown for a number of other proteins (Reaves et al., 1993; Molloy et al., 1994). The third transmembrane domain of ATP7A functions as a *trans*-Golgi network targeting signal (Francis et al., 1998). A common endocytic signal for recycling of membrane proteins from the plasma membrane to the *trans*-Golgi network is a C-terminal dileucine motif, which is also required for ATP7A (Trowbridge et al., 1993; Petris et al., 1998). However, these Golgi targeting signals have not been confirmed to date in other Golgi transporters (Abeijon et al., 1997; Guillen et al., 1998).

Since the first primary sequence of a lysosomal transport protein (cystinosin) became evident only recently, little is known about the targeting signals of these transporters. Cystinosin, which is defective in cystinosis, contains at the C-terminal end the tyrosine-based lysosomal sorting signal (GYXXZ motif, where Z is an amino acid with a bulky hydrophobic group) (Town et al., 1998). This motif is present in several lysosomal membrane glycoproteins with 1 or 4 transmembrane domains (i.e. lamp1, lamp2, and limp I) (Hunziker and Geuze, 1996). A different motif for lysosomal targeting is a dileucine-sorting signal, present in limp II which has 2 transmembrane domains. The lysosomal delivery of these lysosomal membrane glycoproteins can occur via two distinct

pathways: indirectly via the plasma membrane and endocytosis, or directly from the *trans*-Golgi network to the lysosome (Hunziker and Geuze, 1996; Marks et al., 1997). The pathway via the plasma membrane and subsequent endocytosis involves the interaction of the tyrosine- and dileucine-based sorting signals with a clatrin adaptor protein (AP-2) at the plasma membrane. This results in internalization, delivery to early endosomes, sorting to a late endosomal compartment and finally to lysosomes. The direct transport of lysosomal membrane glycoproteins from the *trans*-Golgi network to endosomes and lysosomes occurs via Golgi-derived clathrin-coated vesicles and involves the interaction of the sorting signals with another adaptor protein, AP-3 (Le Borgne et al., 1998). Whether these targeting mechanisms for lysosomal membrane glycoproteins are also involved in lysosomal transport proteins (multispanning membrane proteins) remains to be resolved.

## Transport mechanisms

Membrane transport proteins usually exhibit specificity for a specific molecule or a group of closely related molecules, as was also discussed for the lysosomal membrane transporters in Chapter 1.2. The translocation of solutes across different membranes can occur by several mechanisms (Mitchell, 1967; Saier, 1998). Based on these mechanisms membrane transport proteins can functionally be classified as channels or carriers.

1) **Channel proteins** form aqueous pores across the membrane lipid bilayer through which specific solutes (mostly inorganic ions of appropriate size and charge, but also water, glycerol or urea) can diffuse. They allow solutes to cross the membrane only passively (passive transport/facilitated diffusion) down their electrochemical gradient until equilibrium has been reached. The binding sites of channels are present at both sites of the membrane and are accessible to ions at the same time, without undergoing a conformational change. Channels transport often at very high rates (up to $10^8$ ions/sec), but they are not continuously open. They open in response to either a change in the voltage across the membrane (voltage-gated channels) or the binding of a signaling molecule (ligand-gated channels). The signaling ligand can either be a neurotransmitter, an ion, a nucleotide or a GTP-binding regulatory protein.

2) **Carrier proteins** bind the specific solute that they transport and undergo a conformational change in order to transfer the solute across the membrane. In contrast to channels, transport by carrier proteins can be either passive or active. Carrier proteins that pump hydrophilic substrates across the membrane against their electrochemical gradient are called **primary active transporters** (or **pumps**). Their transport is driven by energy directly provided by a metabolic energy source like ATP. Other carrier proteins are not directly driven by an energy source. Instead, they are coupled to the flow of an ion down its electrochemical gradient (**co-transport**), either in the same direction (**symport**) or in the opposite direction (**antiport**). In this case, transport is activated as long as the ion gradient is present. Since the gradient of the driving ions is usually maintained by an

independent primary active pump, these mechanisms are also called **secondary active transport**. Carrier proteins that simply transport a single solute by facilitated diffusion are called **uniporters**.

## Secondary and tertiary structures

Two principle structural motifs occur in membrane transport proteins: a **β-barrel** structure, exemplified by the porins in the (outer) membranes of bacteria, mitochondria, and eukaryotic chloroplasts (Cowan et al., 1992; Mannella, 1992; Nikaido, 1992) and a structure consisting largely of several parallel membrane-spanning **α-helices** (spanners), established for most of the transport proteins. α-helical and β-strand transmembrane elements can also occur together (Uwin, 1992; Hucho et al., 1996). The α-helix and the β-sheet are the two most common folding patterns of proteins. Transport proteins primarily of transmembrane α helical structural elements are found in the cytoplasmic membranes of bacteria and eukaryotes, or in the membranes of eukaryotic organelles (Saier, 1998; Paulsen, 1998a). The basic structural unit of most classes of transport proteins of both the carrier and channel types consist of **six transmembrane α helices,** occurring most frequently in duplicate or quadruplicate (Fig. 3) (Saier, 1994). For example, members of one of the largest transporter families, the major facilitator superfamily (see below) have been shown to have a 12 spanner structure (Fig. 3C) (Maloney, 1990;Saier and Reizer, 1991;Pao et al., 1998). Alternatively, the voltage-gated ion channels possess four units, each of six transmembrane α helices, creating a total of 24 transmembrane helices (Fig. 3A). Only few transport proteins have a different number of spanners per unit (from 3 to 8 and possibly up to 14) (Buhr and Erni, 1993; Landry et al., 1993; Smith et al., 1993). Obviously, the most direct way to learn about transmembrane segments is by high resolution structure determination. Today, the structures of a growing number of integral membrane proteins have been elucidated by electron and X-ray crystallography and $^{1}H$ nuclear magnetic resonance (NMR)(von Heijne, 1997). The determination of the three-dimensional structure of membrane proteins is still very difficult and has been achieved for only a few transport proteins (Cowan et al., 1992; Uwin, 1992;McDermott et al., 1998;Doyle et al., 1998). Topological predictions based on hydropathy analyses of the primary sequence of transport proteins, together with the "positive inside" rule of von Heijne (1992) have been very useful for obtaining structurally relevant information.

## Classification of transport proteins

In the last couple of years impressive progress has been made in the sequencing of the entire genomes of both prokaryotic and eukaryotic organisms. Computer-aided sequence analysis has become an essential molecular biological tool for the estimation of the structures, functions, and evolutionary relationships of proteins. Several available genomes have been analyzed for genes encoding established and putative transport proteins. (Saier and Reizer, 1991; Nikaido and Saier, 1992; Paulsen et al., 1998a and b).

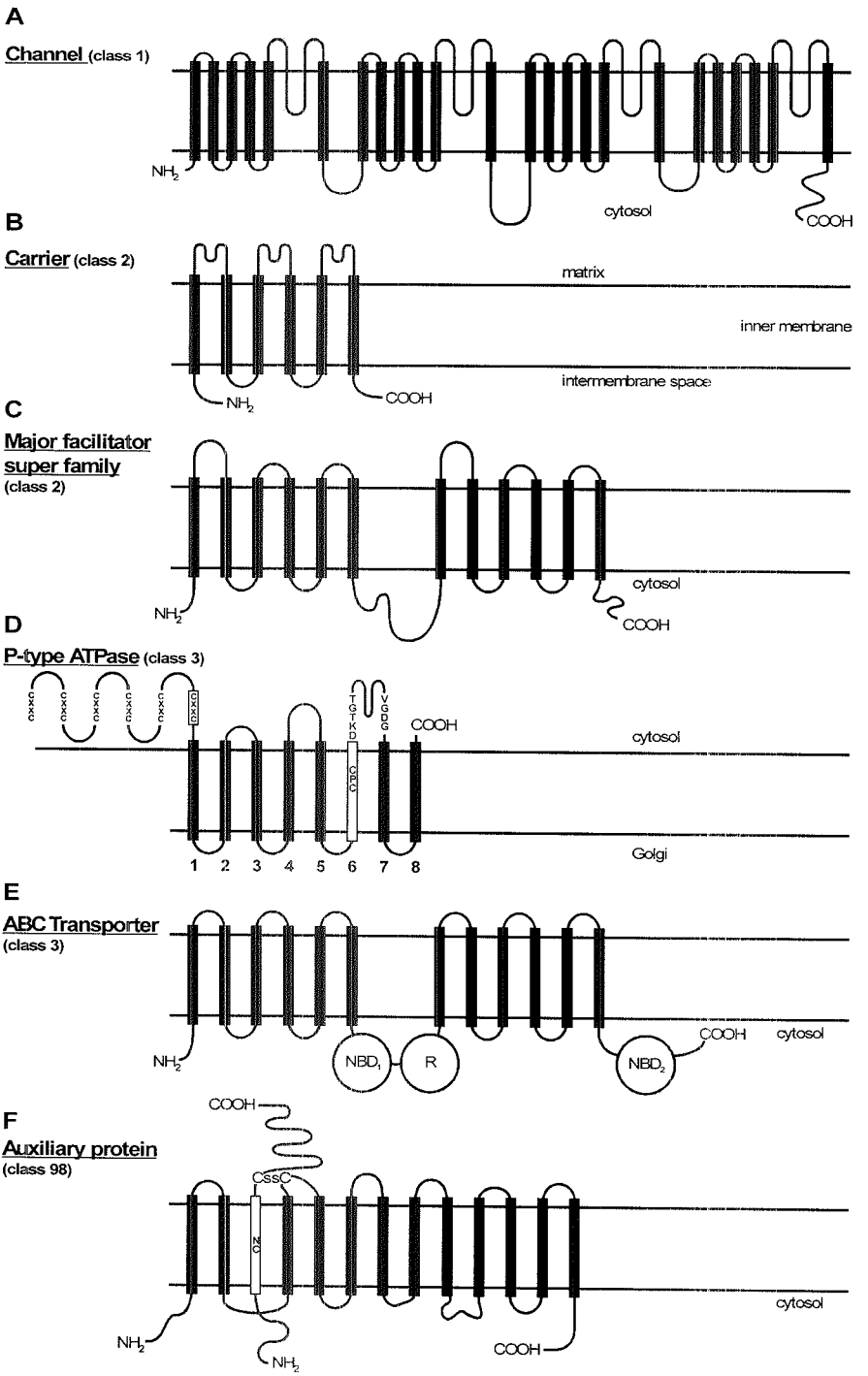


Fig. 3. Topological models of transport proteins from several major transporter families of different classes. A, voltage-gated $Ca^{2+}$ channel; B, mitochondrial carnitine carrier; C, glucose transporter; D, Menkes protein (ATP7A); E, CFTR; F, heterodimeric amino acid transporter (4F2hc).

On the basis of sequence similarities distinct families for transport proteins have been described (Saier and Reizer, 1991;Saier, 1994). These families comprise of both prokaryotic and eukaryotic members. They share structural and functional characteristics which suggest that they form a superfamily, i.e. share a common evolutionary origin. Apparently, substrate specificity is often well conserved through evolution.

Recently, a systematic approach has been devised to classify transporters on the basis of function and evolutionary trait (phylogeny) (Saier, 1998 and http://www-biology.ucsd.edu/~msaier/transport). The first level of classification of the transport systems is the basis of transport mode and energy coupling mechanisms (i.e. function); the second level of classification is based on phylogenetic families; the third level is based on phylogenetic subfamilies or clusters, and the final level is based on substrate specificities. This system is termed the 'Transport Commission' (TC) system (Saier, 1998). Most of the sequenced eukaryotic transport proteins possess homologous prokaryotic counterparts and *vice versa*, although some are restricted to just one of these domains.

Classification according to transporter type and energy source is as follows (for a recent update: see http://www-biology.ucsd.edu/~msaier/transport; see also Table 1 for a summary of the largest families occurring in bacteria, archaea and eukarya): Channel-type transporters (class 1), Carrier-type transporters (uni-, sym- and antiporters) (class 2), Pyrophosphate bond (ATP, GTP) hydrolysis-driven active transporters (class 3), Phosphotransferase systems (group translocators) (class 4), Decarboxylation-driven active transporters (class 5), Oxidoreduction-driven active transporters (class 6), Light-driven active transporters (class 7), Mechanically driven active transporters (class 8), Outer membrane porins (of β-structure) (class 9), Methyltransferase-driven active transporters (class 10), Auxiliary transport proteins (class 98) and Transporters of unknown classification (class 99).

Almost all transporter families within class 4 until class 10 are found in prokaryotes and are therefore not discussed here.

*Channel-type transporters*

Channel-type transporters (class 1) are ubiquitously found in all types of organisms from bacteria to higher eukaryotes. They include members of the large Major Intrinsic Protein (MIP) family of aquaporins and glycerol facilitators (Reizer et al., 1993a; Park and Saier, 1996) as well as members of several ion channel protein families (e.g. $K^+$, $Na^+$, $Ca^{2+}$, $Cl^-$ channels) (Alexander and Peters, 1997; Jentsch et al., 1995). Structurally, these channel proteins consist largely of α-helical spanners, although β-strands may also be present and may even comprise the channel, as is the case for the acetylcholine receptor (Hucho et al., 1996). However, outer membrane porin-type channel proteins (consisting exclusively of β-strands) are excluded from this class and are instead included in a separate class (class 9). Genetic defects in different voltage-gated channels can cause

**Table 1. The major transport protein families of each class, identified in bacteria, archaea, and eukarya.**

| Transport protein family | Abbreviation | No. of TMSs | Multi-component | Substrate(s) | Distribution* | Example of genetic disorder |
|---|---|---|---|---|---|---|
| **Class 1. Channel proteins** | | | | | | |
| Major intrinsic protein | MIP | 12 or 24 | - | glycerol, water | BEA | |
| Voltage-gated ion channel | VIC | 24 | + | potassium, calcium | BEA | migraine, epilepsy |
| **Class 2. Carrier type transporters (uni-, sym-, antiport)** | | | | | | |
| Major facilitator superfamily | MFS | 12 or 14 | - | sugars, drugs, metabolites, neurotransmitters, nucleosides carboxylates, organic and inorganic anions, organic cations | BEA | carnitine deficiency chondrodysplasia |
| Amino acid-polyamine-choline | APC | 12 | - | amino acids, polyamines, choline | BEA | cystinuria, Hartnup disorder |
| Mitochondrial carrier | MCF | 12 | - | ADP/ATP, phosphate, carnitine | E | oxidative phosphorylation diseases |
| **Class 3. ATP-driven active transporters** | | | | | | |
| ATP-Binding Cassette | ABC | 12 | + | sugars, amino acids, drugs, metal ions, peptides, vitamins, proteins | BAE | cystic fibrosis |
| $H^+$- or $Na^+$ -translocating F-type, V-type, and A-type ATPase | F-ATPase | 4-8 | + | $H^+$ , $Na^+$ | BAE | renal tubular acidosis |
| Cation-translocating P-type ATPase | P-ATPase | 6-8 | + | sodium, calcium, metal ions | BAE | Menkes and Wilson disease |
| **Class 4. Phosphotransferase systems** | | | | | | |
| PTS Glucose-glucoside | Glc | 12 | + | glucose, sucrose, β-glucosides *N*-acetylglucosamine | B | |
| **Class 5. Decarboxylation-driven active transporters** | | | | | | |
| $Na^+$- transporting carboxylic acid decarboxylase | NaT-DC | 11 | + | carboxylic acids | B | |

| Transport protein family | Abbreviation | No. of TMSs | Multi-component | Substrate(s) | Distribution* | Example of genetic disorder |
|---|---|---|---|---|---|---|
| **Class 6. Oxidoreduction-driven active transporters** | | | | | | |
| Proton-translocating NADH dehydrogenase | NDH | | + | proton | BE | Oxidative phosphorylation |
| Proton-translocating Quinol:Cytochrome c reductase | QCR | | + | proton | | diseases " |
| Proton-translocating cytochrome oxidase | COX | | + | proton | | " |
| **Class 7. Light-driven active transporters** | | | | | | |
| Ion-translocating Bacteriorhodopsin | BR | 7 | + | protons, chloride | A | |
| **Class 8. Mechanically driven active transporters** | | | | | | |
| $H^+$ - or $Na^+$ -translocating bacterial flagellar motor | Mot | 1-4 | + | protons, sodium | B | |
| **Class 9. Outer membrane porins, β-structure** | | | | | | |
| General bacterial porin | GBP | β-barrel | - | cations, anions, sugar, phosphate | BE | |
| Mitochondrial and plastid porin | MPP | β-barrel | - | | | |
| **Class 10. Methyltransfer-driven active transporters** | | | | | | |
| $Na^+$-transporting methyltetrahydro-methanopterin:Coenzyme M methyltransferase | NaT-MMM | | - | sodium | A | |
| **Class 98. Auxiliary transport proteins** | | | | | | |
| rBAT family of putative transport accessory proteins | | 1 or 4 | + | amino acids | E | cystinuria |

*B, Bacteria; A, Archeae; E, Eukarya.

various forms of neurological disorders, like migraine, ataxia, and epilepsy, renal diseases, like Liddle syndrome or nephrogenic diabetes insipidus, and deafness (Kellenberger et al., 1998; Steinlein, 1998; Terwindt et al., 1998; Kubisch et al., 1999; Matsumura et al., 1999).

*Carrier-type transporters*

Carrier-type transporters (class 2) represent the largest and most diverse category of transporters. Almost 50% of the families are bacteria-specific and many of them are found in *E.coli* (Paulsen et al., 1998a). Of the currently 70 families, the major facilitator superfamily (MFS) and the amino acid-polyamine choline family (APC) are the most represented families (Henderson and Maiden, 1990; Griffith et al., 1992; Reizer et al., 1993b; Pao et al., 1998). The MFS is a very old, large and diverse superfamily that includes several hundreds of sequenced members. Members of the MFS transport small solutes, often in response to ion gradients and are recently classified into 25 distinct families (Pao et al., 1998; recent update http://www-biology.ucsd.edu/~msaier/transport). Most proteins are 400-600 amino acids in length and possess either 12 or 14 putative transmembrane α-helical spanners Fig. 3C). The APC family utilizes either a proton symport mechanism, a solute:solute antiport mechanism, or both, depending on physiological conditions and the carrier under consideration (Reizer et al., 1993b). This family resembles MFS in possessing two structural units, each consisting of six putative transmembrane α-helices.

One of the families of class 2 specifically found in eukaryotes is the mitochondrial carrier family (Fig. 3B), including some carriers involved in oxidative phosphorylation (e.g. adenine nucleotide translocator and phosphate carrier) and some substrate carriers (e.g. 2-oxoglutarate carrier and carnitine-acylcarnitine carrier) (Kuan and Saier, 1993). A deficiency in the heart/muscle isoform of the adenine nucleotide translocator (ANT1) results in mitochondrial myopathy and cardiomyopathy, as has been demonstrated in mice lacking the ANT1 (Graham et al., 1997). Mutations in the carnitine-acylcarnitine carrier cDNA have been established in patients with carnitine-acylcarnitine translocase deficiency, a mitochondrial fatty acid oxidation disorder (Huizinga et al., 1997, 1998; Roe and Coates, 1995).

*Pyrophosphate bond (ATP,GTP) hydrolysis-driven active transporters*

This class of transporters includes systems which hydrolyze pyrophosphate, or the terminal pyrophosphate bond in ATP, or another nucleoside triphosphate to drive the active uptake and/or extrusion of a solute. Five major types of solute-transporting ATPases are found ubiquitously (P-type, F-type, V-type, A-type, and ABC-type). The P-type ATPases make up one superfamily, the F-, V- and A-types together make up a second superfamily, and the ABC- types probably form a huge and diverse superfamily.

P-type ATPases catalyze the uptake and/or efflux of cations. This superfamily comprises 9 subfamilies, which in eukaryotes are present in the plasma membrane and organellar membranes (Fagan and Saier, 1994; recent update http://www-biology.ucsd.edu/~msaier/transport). Many eukaryotic P-type ATPases are multicomponent. They have a large catalytic α-subunit that hydrolyses ATP, contains the aspartyl phosphorylation site and catalyses ion transport and an auxiliary β-subunit that probably facilitates proper insertion of the α-subunit into the membrane, to allow proper targeting to a subcellular membrane site and to stabilize the catalytic α-subunit (Geering et al., 1989). Examples are $Na^+/K^+$-ATPases, $Ca^{2+}$-ATPases, $H^+$-ATPases, $Mg^{2+}$-ATPases, $Cu^{2+}$-ATPases and other metal ion ATPases for Zn, Cd, Pb, Co and Ni (Saier, 1998). A copper ATPase, ATP7A (Fig. 3D), is deficient in patients with Menkes disease, leading to a copper insufficiency (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). ATP7B, a homologue of ATP7A and also encoding a copper ATPase, is deficient in Wilson disease (Bull et al., 1993). In this disease copper accumulates mainly in the liver due to a failure to excrete copper into the bile and to incorporate copper into ceruloplasmin. The exact localization of the Wilson protein is, however, still debated, and has been assigned to the *trans*-Golgi network, plasma membrane, a cytoplasmic vesicular compartment, and mitochondria (Hung et al., 1997;Yang et al., 1997;Nagano et al., 1998;Lutsenko and Cooper, 1998). Recently, we have characterized a new lysosomal heavy metal transporter (Publication IV). However, this P-type ATPase is different from the ATP7B copper transporter, since normal transport activities in lysosomal membrane vesicles of Long Evans Cinnamon rats, an animal model for Wilson disease, were observed.

F-, V- and A-types of ATPases catalyze the translocation of $H^+$ or $Na^+$. A-type ATPases are found in archaea (a recently recognized class of microorganisms between the bacteria and eukarya). F-type ATPases are located at the inner membrane of mitochondria and chloroplasts (Blair et al., 1996). V-type ATPases are present in vacuoles (e.g. lysosomes) and on apical membranes of acid-secreting cells, like epithelial cells of kidney and inner ear (Finbow and Harrison, 1997). V-type $H^+$-ATPases pump protons against an electrochemical gradient, whereas F-type ATPases reverse the process, synthesizing ATP (therefore also called F-ATPsynthases). Recently, it has been shown that mutations in a V-type $H^+$-ATPase cause a human disease, renal tubular acidosis with sensorineural deafness (Karet et al., 1999).

Only one transporter superfamily is larger and more diverse in function than the MFS, and that is the ABC superfamily. This superfamily contains at least 40 families of transporters which have specificity for similar kinds of small molecules as the MFS, but in addition they can transport macromolecules, such as proteins, complex carbohydrates and lipids (Saier, 1998). They fall into three major phylogenetic and functional categories: prokaryotic uptake systems, prokaryotic efflux systems and eukaryotic efflux systems. Currently, no eukaryotic ABC-type uptake system is known. The ABC transporters are

also multicomponent systems with sizes of over 1000 amino acids each and usually possess a 12 (6 + 6) TMS topology. Well-characterized members include the maltose uptake permease of E. coli, the multidrug resistance pump (MDR) and the cystic fibrosis transmembrane conductance regulator (CFTR) (Fig. 3E). Mutations in the CFTR gene causes cystic fibrosis, the most common life-shortening autosomal recessive disorder in caucasian populations, characterized by chronic pulmonary disease and often exocrine pancreatic insufficiency (Rosenstein and Zeitlin, 1998).

*Auxiliary transport proteins*

Proteins that function with or are complexed to known transport proteins are included in the category of auxiliary transport proteins (class 98). Examples are energy coupling and regulatory proteins that do not actually participate in transport, like the β-subunit of the voltage-gated $K^+$channel. Analogous to this phenomenon are the rBAT (related to $b^{0,+}$ amino acid transporter) and 4F2hc (cell surface glycoprotein 4F2 heavy chain) glycoproteins which are part of the heterodimeric structure of amino acid transporters (Moskovitz et al., 1994; Palacin et al., 1998; Mastrobernandino et al., 1998). Expression studies of the rBAT and 4F2hc genes in *Xenopus laevis* oocytes induced amino acid transport. Mutations in the rBAT gene cause cystinuria type I, a disease characterized by a defective renal and intestinal reabsorption of cystine and dibasic amino acids. Their function as a heterodimer was recently elucidated, when the light chain of human 4F2hc (E16 or hAmAt-L-lc) was identified and the resulting human heterodimeric complex type L amino acid transport mediated (Mastrobernandino et al., 1998). A model for the membrane topology of this type L amino acid transporter was presented (Fig. 3F). Parallel studies by Palacíns group identified a different light chain ($y^+$LAT-1), which forms with 4F2hc the heterodimeric transporter with $y^+$L-amino acid transport characteristics (Torrents et al., 1998). The gene for $y^+$LAT-1 is mutated in lysinuric protein intolerance (LPI), an inherited disorder of cationic amino acid transport (Torrents et al., 1999; Borsani et al., 1999).

*Transporters of unknown classification*

Putative transport proteins of which the exact function is not yet known are temporarily classified as transporters of unknown classification (class 99).

## Evolution

As shown in Table 1, many transport proteins of different classes share a common topology, consisting of a subunit of six transmembrane α-helices which can be repeated two or four times (Saier, 1994). This led to the assumption that all transport proteins have evolved from a common ancestor during evolution, from prokaryotes on (Henderson and Maiden, 1990; Maloney, 1990). However, Saier's group has conducted extensive phylogenetic analyses of transport protein families and suggested that some of the

families have evolved independently of each other. Six transmembrane domains can be formed by a duplication of three-spanners (MIP family), which happened possibly 2.5 billion years ago (Reizer et al., 1993b). The major facilitator superfamily probably arose independently of the MIP family more than 3.5 billion years ago by the intragenic duplication of a six-spanner precursor to give proteins containing 12 transmembrane α-helical spanners (Pao et al., 1998). The mitochondrial carrier family (MCF) arose independently of those two ubiquitous families of transporters by triplication of a precursor encoding a two-spanner. The time of emergence of the MCF precursor polypeptide-encoding gene is estimated at about 1.5 billion years ago, the time when mitochondria first appeared in eukaryotes; hence then are not derived from proteins present in the prokaryotic progenitors of mitochondria (Saier, 1998).

Comparison of the established or putative topologies of subunits of channel protein families with those of carrier families, revealed that channel protein subunits usually possess two to six transmembrane domains, while carrier protein subunits usually possess 10-14. In addition, channel proteins usually consist of oligomers, while the carriers have often been found to be monomers. Although exceptions exist for both classes of proteins, these two classes seem to have different structural requirements which allow them to be distinguished on the basis of topological features alone (Saier, 1998).

Some transport modes and energy-coupling mechanisms appear to occur ubiquitously in all of the three domains (bacteria, archaea, eukarya), while others are restricted to one domain (Saier, 1998). Mechanisms which are ubiquitous (i.e. channels, secondary carriers, ATP-driven active transporters) may have arisen early, that is before the divergence of the three domains of life.

The computer programs can not predict whether these families stem from a common ancestor or not. Elucidation of the detailed three-dimensional structures of transmembrane transport proteins and thus of their common or divergent structural features will be required to fully define the relationship between different transmembrane transporters.

## 1.4 Genetic disorders of lysosomal membrane transport

The occurrence of (human) genetic disorders provides an important tool to understand fundamental cellular mechanisms and protein functions. In particular the two inborn errors of metabolism cystinosis and sialic acid storage disease have contributed to the understanding of lysosomal membrane transport systems (Gahl et al., 1984;Mancini et al., 1991;Gahl et al., 1995).

### Cystinosis

Cystinosis is genetically homogeneous but clinically subdivided into three phenotypic types: infantile, adolescent and adult cystinosis. The classical form of cystinosis (infantile or nephropatic cystinosis) occurs in 95% of the patients, with an incidence of approximately 1 in 200,000 live births. The basic defect is an impaired function of the lysosomal cystine transporter, leading to lysosomal accumulation of free cystine. Cystine crystals deposited in the kidney cause a generalized defect in proximal tubule function, leading to impaired reabsorption of small molecules (Fanconi renal syndrome). Patients develop Fanconi renal syndrome in the first year of life. Other clinical features are growth retardation, progressive renal failure within the first decade, and a variety of other complications, including photophobia and corneal erosions due to cystine crystal formation within the eye (Gahl et al., 1995). Milder variants of the classical disease include an intermediate form with late-onset renal disease (adolescent cystinosis), and a benign form with corneal involvement but no renal impairment (adult cystinosis). The different clinical forms of cystinosis are allelic and likely due to mutations within the same gene (Pellet et al., 1988).

New insights into the pathophysiology of the Fanconi syndrome have recently come from *in vivo* and *in vitro* studies in rats (Ben-Nun et al., 1993; Baum, 1998). The Fanconi renal syndrome can be induced in rats by the administration of cystine-dimethyl ester (Foreman et al., 1987). The following abnormalities were observed: a transient decline in ATP content in the renal cortex homogenate, and a decrease in the $V_{max}$ of glucose transport in brush border membrane vesicles. No change occurred in the activity of the $Na^+/K^+$ ATPase. The decrease in ATP was probably due to a low intracellular phosphate concentration in cystine loaded tubules. A reduction in the number of sodium-coupled glucose transporters partially explained the decrease in the $V_{max}$. The reduction in ATP content and sodium-coupled glucose absorption may be the basis for the Fanconi renal syndrome seen in those rats, and is probably one of the mechanisms for the proximal tubular impairment seen in cystinotic patients.

Cystinosis is treated by oral administration of cysteamine bitartrate capsules (Cystagon) as approved by the US Food and Drug Administration in 1994. Already in 1976 the effectiveness of this cystine-depleting agent was shown (Thoene et al., 1976). The drug reduces the intracellular concentration of cystine, and, if used early in the disease and in high doses, can reduce the progress of renal glomerular damage and

improve growth (Gahl et al., 1995). With the recent demonstration of the presence of a lysosomal cysteamine transporter the intracellular route of this drug can be conceived (see Chapter 1.2).

Recently, the gene for cystinosis has been identified (Town et al., 1998). It has been mapped to a 4 cM region on chromosome 17p13 by linkage analysis (The Cystinosis Collaborative Research Group, 1995). Afterwards, this critical region was progressively narrowed (Jean et al., 1996;McDowell et al., 1996; Peters et al., 1997) and several cystinosis patients were found to have deletions in a specific microsatellite marker within the region (Town et al., 1998). Physical mapping near this deletion led to the isolation of the cystinosis gene, CTNS, which is mutated in patients with the infantile type of cystinosis. The most frequent mutation found is a 65 kb deletion, which is detected only in patients of European origin suggesting a founder effect. In addition to the 11 reported small mutations by Town et al. (1998), 18 new mutations in CTNS have recently been reported by Shotelersuk et al. (1998) in an American-based population of cystinosis patients. CTNS mutations are localized in the leader sequence, transmembrane and nontransmembrane regions.

According to a cystinosis clinical severity score, the clinical phenotype of infantile cystinosis correlated with the mutations. Homozygotes for the 65 kb deletion and W138X mutation suffer from a more severe disease than patients with mutations involving the first amino acids prior to transmembrane domains. Those infantile patients have a less severe disease. Mutations responsible for the allelic variants of cystinosis, i.e. adolescent and adult cystinosis, have yet to be identified.

The isolation of the cystinosis gene will have little or no immediate influence on the clinical care of patients, since diagnostic and therapeutic tools are already available for cystinosis. At the moment, pre- and postnatal diagnosis will continue to depend heavily on amniotic fluid cell or leukocyte cystine measurements, but perhaps eventually molecular diagnosis of nephropathic cystinosis will take its place.

### Sialic acid storage disease (SASD)

Salla disease and infantile sialic acid storage disease (ISSD) are clinical phenotypes of the same genetic disorder, sialic acid storage disease (SASD), and are characterized by increased lysosomal accumulation and urinary excretion of free sialic acid due to a defective lysosomal transporter for sialic acid and glucuronic acid (Mancini et al., 1991). Obligate heterozygotes show intermediate transport activities, while patients are totally deficient. The eponym Salla disease refers to the geographically restricted area in northeastern Finland where the first described patients reside (Aula et al., 1979). The disease frequency in the population in this area has been estimated as high as 1:6400 (Aula et al., 1986). Salla disease patients develop psychomotor delay, hypotonia, and ataxia between 3 and 12 month of age. At a later stage, patients show severe psychomotor retardation, impaired speech and growth retardation, but their life expectancy is only slightly reduced (Gahl et al., 1995). The clinical features of ISSD resemble those of Salla

disease, but are much more severe. Disease manifestations are present already at birth and include enlarged liver and spleen, coarse facial features, and developmental delay. Growth is poor and mental and motor retardation severe and patients die in the first years of life. ISSD appears to be less frequent than Salla disease and has no ethnic prevalence. In addition to the two main phenotypes, intermediate forms have also been described with clinical features of both Salla disease and ISSD (Baumkötter et al., 1985;Ylitalo et al., 1986;Mancini et al., 1992b).

The diagnosis of SASD depends mainly on the clinical findings, the biochemical demonstration of elevated urinary excretion of free sialic acid by thin layer chromatography, and the histological presence of enlarged lysosomes in various types of cells and tissues (Gahl et al., 1995). The difference in severity between Salla disease and ISSD is not only clinically visible, but is also reflected in the amount of free sialic acid excreted in the urine and accumulated in tissues as the levels in ISSD patients are much higher than in Salla disease patients.

Excessive urinary excretion of free sialic acid is also characteristic for patients with sialuria. This rare disorder is characterized by varying degrees of developmental delay, enlarged liver and spleen, and coarse facial features (Gahl et al., 1995). The sialic acid overproduction and excretion in sialuria is caused by a defective feedback inhibition in the synthesis of sialic acid (Weiss et al., 1989). Sialuria can be distinguished from SASD in the intracellular site of the accumulated free sialic acid, which is cytosolic in sialuria and intralysosomal in SASD (Seppala et al., 1991).

The clinical difference between Salla disease and ISSD is not reflected in the transport activities. Both phenotypes are almost completely devoid of proton-dependent transport of sialic acid and glucuronic acid in lysosomal membrane vesicles of cultured fibroblasts. An explanation for the lower levels of sialic acid storage in Salla disease may be the presence of residual transport activity *in vivo*. However, the present transport assay lacks the sensitivity to demonstrate such slight differences. So far, only slightly increased amounts of glucuronic acid have been found in cultured fibroblasts of SASD patients (Blom et al., 1990). Investigations on the urinary excretion of glucuronic acid in SASD patients have never been performed, probably due to methodological difficulties in glucuronic acid determination.

Transport activity measurements are complex and time-consuming and therefore not suitable for a biochemical diagnosis of patient or heterozygote detection. A more feasible method could be the measurement of free sialic acid levels in the polymorphonuclear leucocyte subpopulation (Mancini et al., 1992b). Since the gene for Salla disease has been assigned to chromosome 6q14-q15 by linkage analysis (Haataja et al., 1994a; Schleutker et al., 1995a), carrier detection and prenatal diagnosis can be performed by haplotype analysis of linked microsatellite markers (Schleutker et al., 1996). ISSD represents the same allelic disorder as Salla disease (Schleutker et al.,

1995b). Subsequently, the critical DNA region for the SASD gene was refined to a 200 kb chromosomal region by analyses of extended haplotypes (Leppänen et al., 1996).

The pathogenesis of SASD and the role of sialic acid and glucuronic acid (and perhaps other compounds as well) is still unclear. Recent investigations of the ganglioside metabolism in Salla disease fibroblasts suggest that the lysosomal accumulation of sialic acid reduces the turnover rate, not only of gangliosides and other membrane-bound sialoglycoconjugates, but also of sphingolipids in general, including ceramides (Berra et al., 1995; Chigorno et al., 1996; Pitto et al., 1996).

### Potential lysosomal membrane transport disorder

A few studies suggest that a third genetic disorder, **cobalamin F disease**, is due to a defective lysosomal transport system. Fibroblast studies indicated that patients accumulate unmetabolized, non-protein bound cobalamin (vitamin $B_{12}$) in their lysosomes after dissociation from a transcobalamin II-vitamin $B_{12}$ complex in the lysosome (Rosenblatt et al., 1985;Vassiliadis et al., 1991). Shortage of vitamin $B_{12}$ in the cytosol will result in the impaired synthesis of the two derivatives of cobalamin, methyl cobalamin and 5'-deoxyadenosyl cobalamin. These are essential cofactors for the cytosolic methionine synthase and the mitochondrial methylmalonyl CoA mutase, respectively enzymes important in amino acid metabolism. Cobalamin F disease presents clinically as homocystinuria combined with methylmalonic aciduria and sudden death within the first year of life (Kapadia, 1995;Fowler, 1998). A specific transport system for vitamin $B_{12}$ has been characterized in the lysosomal membrane, but it is unknown whether this transporter harbours the primary defect in cobalamin F disease (Idriss and Jonas, 1991).

### Other potential disorders of lysosomal membrane function

There are at least two other congenital disorders, Niemann-Pick disease type C and Batten disease (juvenile neuronal ceroid lipofuscinosis), which are characterized by a lysosomal storage and with a possible involvement of membrane components.

**Niemann-Pick disease type C** is a severe genetic disorder, characterized by lysosomal accumulation of low-density lipoprotein (LDL)-derived cholesterol and sphingomyelin and by progressive degeneration of the nervous system (Pentchev et al., 1995;Liscum and Klansek, 1998). Several lines of evidence indicate that the intracellular trafficking of free cholesterol from lysosomes to other cell membranes is defective (Sokol et al., 1988;Neufeld et al., 1996; Shamburek et al., 1997). Linkage analysis has led to the recent cloning of the NPC1 gene on human chromosome 18 (Carstea et al., 1997). NPC1 predicts a 142 kDa integral membrane protein with 13-16 possible transmembrane regions and contains at the C-terminus a dileucine motif, which may serve as a lysosomal targeting sequence (Hunziker and Geuze, 1996). It exhibits homology with mediators of

cholesterol homeostasis, which suggests that NPC1 may play a role in the endocytic pathway of cholesterol. It contains domains in its N-terminus that are critical for the mobilization of cholesterol from lysosomes (Watari et al., 1999). In contrast, recent investigations in a mouse model of Niemann-Pick disease type C have established an early peroxisomal deficiency, affecting a number of functions (Loftus et al., 1997; Schedin et al., 1997). Moreover, treatment with appropriate peroxisomal inducers restored both the peroxisomal and lysosomal functions (Schedin et al., 1998). Further studies are required to clarify whether this disease belongs to the lysosomal transport disorders.

**Batten disease** (also known as juvenile neuronal ceroid lipofuscinosis or Spielmeyer-Vogt-Sjögren disease) is the most common (incidence 1:12.500) recessively inherited neurodegenerative disorder of childhood. It is characterized by loss of vision, epilepsy and progressive mental deterioration, and leads to early death, usually in the second or third decade. Diagnostic criteria include the presence of lipopigment granules in both neural and non-neural cells on electron microscopy. The major protein component of the abnormal deposits is subunit c of mitochondrial ATP synthase and is localized in lysosomes (Hall et al., 1991;Palmer et al., 1997). CLN3, the gene involved in Batten disease, maps to chromosome 16p12.1 and is predicted to encode a 438 amino acid protein with no homology to other known human genes (The International Batten Disease Consortium, 1995). The high evolutionary conservation of homologues genes in *S. cerevisiae*, *C. elegans*, mouse and dog suggest that the protein is involved in some basic eukaryotic cellular function. Based on hydrophobicity plots, CLN3 protein has been predicted to have six transmembrane domains, like many transport proteins. The exact localization of the CLN3 protein in the cell is debated at the moment. Immunofluorescence microscopy studies showed partial localization both in the lysosomal membrane and in the Golgi membrane (Järvelä et al., 1998;Kremmidiotis et al., 1999). The putative yeast homologue of Cln3p, Btn1p, is located in the yeast vacuole, the yeast equivalent of the mammalian lysosome (Croopnik et al., 1998).

There is no reason to assume that this list of (potential) lysosomal transport disorders is all inclusive. Additional disorders related to a lysosomal transport dysfunction might exist and must be searched for among patients with unexplained lysosomal storage diseases.

## Chapter 2 DISCUSSION OF THE EXPERIMENTAL WORK

**2.1 Purification of the lysosomal sialic acid transporter**

**2.2 Functional characterization of the lysosomal sialic acid transporter**

**2.3 Characterization of a novel lysosomal heavy metal ion transporter**

## 2.1 Purification of the lysosomal sialic acid transporter

The difficulties in the purification of a membrane protein are to find a suitable detergent to extract most of the protein out of its natural membranous environment, and to find conditions in which the protein remains stable and maintains its biological activity during the purification procedure. A prerequisite is to have an assay which allows the determination of the protein's biological activity after it has been solubilized. Our group has previously developed a method to reconstitute the lysosomal sialic acid transporter into liposomes in a biologically active form (Mancini et al., 1992a). With this functional reconstitution system we were able to follow the fractionation and purification of the solubilized lysosomal sialic acid transporter.

For the purification of the sialic acid transporter we used classical chromatographic techniques. In practice, most of the problems arising in membrane protein chromatography are due to the presence of a detergent. Detergent molecules may alter the chromatographic matrix by binding to it and the size of the micelles may change the chromatographic behavior of a protein.

In **publication I**, we describe the purification of the lysosomal sialic acid transport protein, based on its biological activity (Fig. 4). This represents the first report on the purification of a functionally active lysosomal membrane transporter. The approach was based on chromatographic techniques previously used for mitochondrial carboxylate transporters. For instance, hydroxyapatite chromatography has been very useful in the isolation of a number of mitochondrial transport proteins, whereas ion exchange and affinity chromatography have been useful in the purification of other transport proteins as well as some lysosomal membrane proteins (Kyouden et al., 1992; Kim et al., 1993; Mandon et al., 1994; Palmieri et al., 1994; Schulte and Stoffel, 1995;).

Highly purified lysosomal membrane vesicles isolated from rat livers were used as a starting material, since the sialic acid transporter was successfully solubilized and reconstituted from this source. After solubilization, the lysosomal sialic acid transport protein was purified to apparent homogeneity by a combination of in total 5 different chromatographic columns. We used successively hydroxyapatite, Lentil lectin, DEAE-Sephacel anion exchange, a second time hydroxyapatite, and finally MonoQ anion exchange chromatography. The first purification step consisted of several small columns of dry hydroxyapatite material (Bisaccia and Palmieri, 1984, Bisaccia et al., 1988). The Lentil lectin column separated the carrier from a number of major lysosomal membrane glycoproteins (Fukuda, 1991). These glycoproteins bound to the Lentil lectin column, whereas the sialic acid transporter did not and was found in the column flow-through. This flow-through fraction was subsequently applied to a DEAE-Sephacel anion exchange column. With 100 mM NaCl, 12 % of the total transport activity was eluted (see publication I, purification table). The second hydroxyapatite column was used under different conditions of pH and sodium chloride concentrations, thereby retaining different

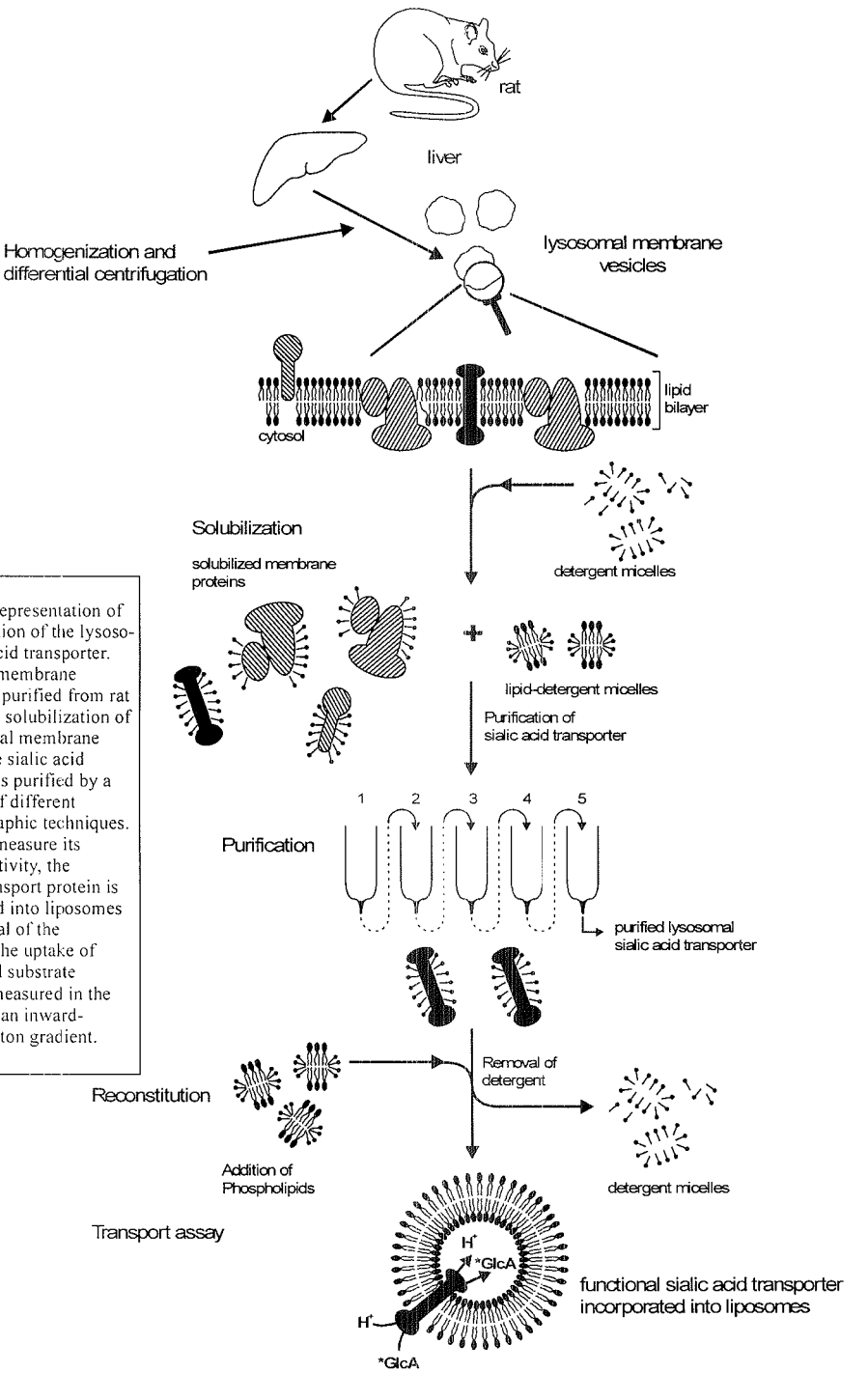


Fig. 4.
Schematic representation of the purification of the lysosomal sialic acid transporter. Lysosomal membrane vesicles are purified from rat livers. After solubilization of the lysosomal membrane proteins, the sialic acid transporter is purified by a procedure of different chromatographic techniques. In order to measure its transport activity, the purified transport protein is incorporated into liposomes after removal of the detergent. The uptake of radiolabeled substrate (*GlcA) is measured in the presence of an inward-directed proton gradient.

proteins from those retained by the first hydroxyapatite column. This step provided an important purification of the sialic acid transport protein. The fifth and last Mono Q anion exchange column was a very small column of 100 µl, which was attached to a computerized system for micropreparative chromatography (SMART system of Pharmacia Biotech). This system separates proteins with a very high resolution (very small gradient volume and very small fractions).

Analysis of the protein composition of the fractions obtained from the Mono Q column showed that only a 57 kDa protein band correlated with the pattern of transport activity in those fractions, measured after reconstitution. As shown in Fig. 2 of publication I, the silver stained 57 kDa protein band is predominant in the fraction, in which also the highest transport activity could be measured. All other visualized proteins in this fraction could not represent the sialic acid transporter, because they became more prevalent in following fractions, where lower or no transport activity was detected.

Investigations of the properties of the purified sialic acid transporter suggested that this protein is not heavily glycosylated and is not functional as a (homo)dimer or polymer linked by disulfide bridges. Most characterized lysosomal membrane proteins, such as the LAMPs and LIMPs, are densely N-glycosylated (Fukuda, 1991). Although their functions are yet unknown, their secondary structures do not suggest a transport function. An advantage of our reconstitution system is that we use liposomes, which do not contain endogenous active proteins. Another commonly used assay system for transport proteins is *Xenopus laevis* oocytes. This system is especially useful in expression studies of cloned transporter genes, but a disadvantage of the *Xenopus* system is the presence of endogenous proteins. This fact is illustrated by the expression studies of the rBAT and 4F2hc proteins, which are both involved in amino acid transport processes (Bertrán et al., 1992a, b; Tate et al, 1992; Wells et al., 1992a, b). Since both proteins induced amino acid transport in *Xenopus* oocytes, it was thought that rBAT (only 1 or 4 transmembrane domains) and 4F2hc (only 1 transmembrane domain) represented amino acid transporters themselves. However, later it was shown that the induction of amino acid transport was due to association of these proteins with other essential endogenous proteins (Mastroberardino et al., 1998; Torrents et al., 1998). The fact that the purified protein induced glucuronic acid transport after reconstitution into liposomes that do not contain other endogenous proteins, proved that it does not need other proteins for its function.

Several attempts have been made to obtain an N-terminal amino acid sequence of the purified lysosomal sialic acid transport protein by Edman degradation. However, the final amount of the 57 kDa protein was too low to obtain a reliable stretch of amino acids. New, more sensitive techniques, like mass spectrometric sequencing, are currently exploited in order to sequence unknown low abundant proteins (Shevchenko et al., 1996). Such a technique might be helpful in sequencing the sialic acid transporter. As discussed in the next section, extensive functional characterization of the purified protein provided an approach towards selection of functional candidates.

## 2.2 Functional characterization of the lysosomal sialic acid transporter

Elucidation of the functional properties of the sialic acid transporter may provide a better understanding of the sialic acid storage disease and in general the functions of lysosomes. Several functional characteristics of the lysosomal sialic acid transporter are known: 1) transport of sialic acid across the lysosomal membrane is a carrier-mediated process, 2) the mechanism is that of secondary active cotransport of sialic acid with protons and 3) the protein has a substrate specificity for acidic monosaccharides (Mancini et al., 1989;Mancini et al., 1991).

The characteristic kinetic features of a carrier-mediated process are expressed by the Michaelis Menten equation ($V=SV_{max}/[K_m + S]$). A carrier protein has a limited number of specific binding sites. When all these binding sites are occupied (the carrier is saturated) at a given substrate concentration (S), the rate of transport is maximal ($V_{max}$). Besides a characteristic $V_{max}$, each carrier protein has also a characteristic binding constant for its substrate, $K_m$, equal to the concentration of substrate when the transport is half its maximum rate.

Investigations on the substrate specificity of a transporter are performed by *cis*-inhibition and *trans*-stimulation transport studies. In the case of *cis*-inhibition studies, the compound of interest is located at the same site of the membrane as the substrate (in our assay that is radiolabeled glucuronic acid)(Figure 5A). A specific inhibitor can influence the binding of the substrate in a competitive (i.e. the inhibitor competes for the same binding site and may or may not be transported by the carrier) or in a non-competitive mode (i.e. the inhibitor binds elsewhere and specifically alters the structure of the carrier).

To test whether an inhibitor not only interacts at the substrate-binding site but is also actually transported across the membrane, the *trans*-stimulation effect of the inhibitor on the transport of substrate can be investigated. In order to observe a *trans*-stimulation effect experimentally, two transport assay conditions are compared: in one assay the vesicles are prefilled with unlabeled substrate, in the other assay the vesicles are not preloaded (Fig. 5B). In the "preloaded" condition, the unlabeled inhibitor is located at the *trans*-side of the membrane at a concentration above $K_m$, higher than the labeled substrate at the *cis*-side (below $K_m$). This will cause a net substrate flux from *trans* to *cis*. Consequently, the velocity of the process will be determined by the higher concentration present at the *trans*-side. Since a carrier protein has its binding sites alternately available at each side of the membrane, the influx of the radiolabeled substrate will be much faster in the preloaded vesicles compared to the unpreloaded situation.

Previous kinetic studies with the radiolabeled forms of sialic acid and glucuronic acid demonstrated their translocation across the lysosomal membrane via a single transport system. The sialic acid transporter recognizes also galacturonate, the aldonic acids gluconate and galactonate, and the lactons of glucarate (D-saccharic acid-1,4 and 1,3-lactons) (Figure 6) (Mancini et al., 1989, 1991, 1992a). The presence of a carboxyl-

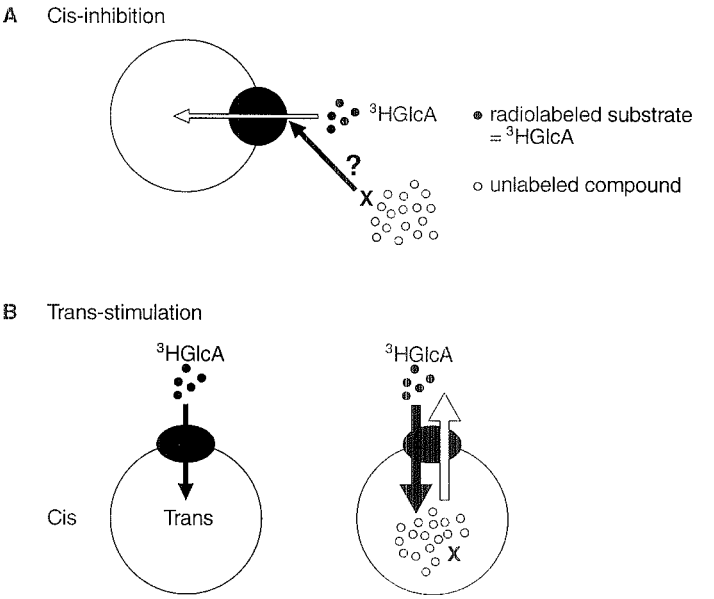


Figure. 5. *Cis*-inhibition (A) and *trans*-stimulation (B) kinetic studies. A: compound of interest is present at the same side of the membrane as the radiolabeled substrate. B: in the preloaded vesicle (right) the influx is much faster than in the not preloaded one (left), due to the efflux of unloaded substrate.

group, but not its position in the molecule (in sialate at C-1, in glucuronate at C-6), and an alcohol group at carbon 2 are important for recognition by the transporter. An hemiacetal ring structure is probably unimportant, since aldonic acids, which do not spontaneously form rings, are also recognized (Mancini et al., 1989).

In **publication I**, we demonstrated the transport of the isomer of glucuronic acid, iduronic acid (Fig. 6) by the lysosomal sialic acid transporter. Iduronic acid has not been tested earlier, because it became only recently commercially available. This uronic acid is another important physiological component of glycosaminoglycans. So far, lysosomal accumulation of iduronic acid in patients with SASD has not been investigated, nor its transport across the lysosomal membrane of patient fibroblasts.

Besides monocarboxylated anionic sugars, the carrier also recognized a small aliphatic non-sugar monocarboxylated anion, pyruvate (Mancini et al., 1989). Pyruvate is a natural precursor in sialic acid biosynthesis. In publication I, we compared the biochemical characteristics of our purified carrier with those of short chain monocarboxylate (like lactate) transporters (MCT1, MCT2, and MCT3)(Garcia et al.,

1994, 1995; Yoon et al., 1997), present in the plasma membrane of various mammalian cells (Poole and Halestrap, 1993). We observed some striking biochemical similarities. Both are proton-coupled transporters and are sensitive to specific inhibitors, like cyano-cinnamates and stilbene disulfonates. Furthermore, both transporters appear to have essential arginine residues at their substrate binding sites. In **publication** I, we

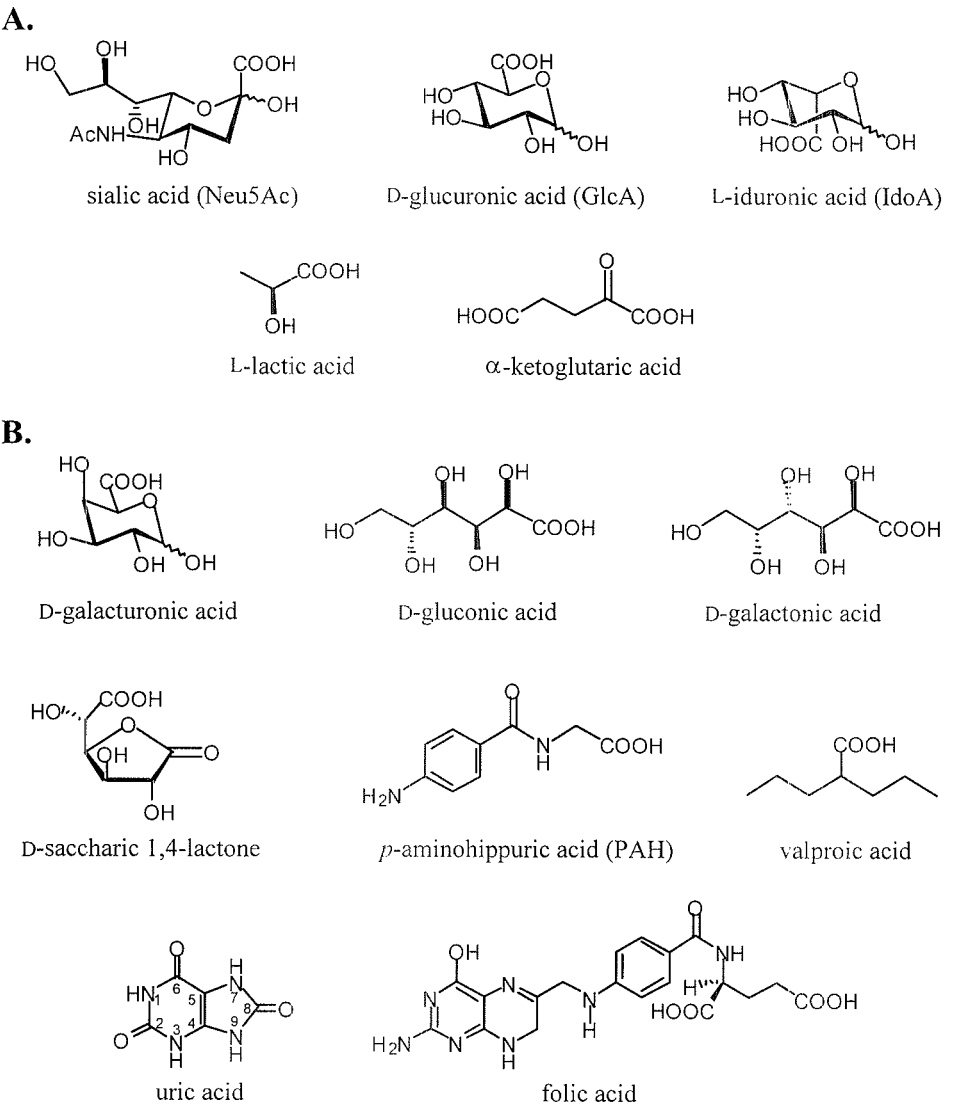


Figure 6. A: structures of the molecules which are transported by the lysosomal sialic acid transporter. B: structures of several molecules which inhibit the uptake of radiolabeled GlcAc.

demonstrated that our carrier also transports L-lactate by *trans*-stimulation and inhibition kinetic experiments with radiolabeled glucuronic acid as well as radiolabeled L-lactate. A common problem in L-lactate transport is the presence of both ionized and unionized forms. The unionized form can pass membranes via simple diffusion and produce changes in membrane potential. To limit the contribution of the unionized form of lactate, transport assays must be performed at lower temperatures (20°C), in the presence of low concentrations of lactate (15 μM) and at a pH above the pK of lactate. Even under these conditions, we observed competition between lactate and sialic acid for the same transport binding site. Apparently, there is an overlap in substrate specificity between MCTs and the sialic acid carrier. Assuming a possible structural homology of the sialic acid carrier with MCTs, we have determined the chromosomal localization of 4 recently cloned MCTs (Price et al., 1998). However, none of them mapped to the known SASD locus on 6q14-q15 and could therefore be excluded as candidate genes for SASD (unpublished results).

The MCTs comprise an ancient family of transporters, which is classified as a subfamily of the major facilitator superfamily (MFS)(see chapter 1.3). Besides the MCT family, the MFS contains also other organic anion transporter subfamilies. Those families in which organic anions are known to be transported by a proton symport mechanism are of special interest. In **publication II**, we demonstrated that several typical substrates for carriers belonging to a number of anion transporter families, were also recognized by the sialic acid transporter. The dicarboxylate α-ketoglutarate, the prototype substrate of organic anion transporters *para*-aminohippurate, the antiepileptic drug valproate, the purine metabolism endproduct urate, and the anionic drug folate were competitive inhibitors, although with different affinities (Fig. 6). Moreover, α-ketoglutarate also showed a *trans*-stimulation effect, indicating that the lysosomal sialic acid transporter can exchange GlcA for α-ketoglutarate. We concluded that the lysosomal sialic acid transporter recognizes not only anionic sugars, but also a wide array of non-sugar anionic compounds (Fig. 6). The structures of the recognized molecules are quite different, ranging from small aliphatic molecules (e.g. lactate) to more complex molecules (e.g. folate). The presence of one or two carboxylic groups appears to be a structural requirement for recognition.

Whether compounds like L-lactate or α-ketoglutarate are physiologically important in lysosomes is not known. Lactate is an important energy source in (neonatal) brain and possible abnormalities in lactate metabolism can be studied by $^{1}$H NMR spectroscopy (Gadian and Leonard, 1996; Medina et al., 1996). Such studies might be interesting in brain from SASD patients in order to understand events of neurodegeneration in this disease. Previous MRI studies showed a defective myelination pattern in Salla disease patients (Haataja et al., 1994b). It would be interesting to study in more detail the role of the lysosomal sialic acid transporter in the transport of the monocarboxylated epileptic drug valproate, its metabolites, and other organic anions by

future experiments with radioactive forms. Also in relation to Salla disease this could be important, since patients with this disease can suffer from epilepsy and might be treated with valproate.

Our transport studies provided the opportunity to select a number of anion transporter families from the major facilitator superfamily to which the sialic acid transporter might belong. The selected families contain proton symporters with an overlap in substrate specificity with the sialic acid transporter. They are the sialate:$H^+$ symporter (**SHS**) family, the organic anion transporter (**OAT**) family, the metabolite:$H^+$ symporter (**MHS**) family, the anion:cation symporter (**ACS**) family and the already discussed monocarboxylate transporter (**MCT**) family, which are all subfamilies of the MFS (Pao et al., 1998). Another interesting family, which is not a member of the MFS, is the acid sugar (gluconate):$H^+$ symporter (**GntP**) family (Saier, 1998). Interestingly, our observation that the sialic acid transporter is a carrier for both sugars and anions is also reflected in some of these families.

The **SHS** family contains besides two putative sialic acid permeases of the prokaryotes *E. coli* and *H. influenza*, also a yeast *S. cerevisiae* homologue, JEN1. The latter was classified on the basis of sequence similarities as a member of this SHS family, while no evidence for sialic acid transport has ever been presented. However, JEN1 is known to function as a carboxylic acid transport protein, transporting L-lactate (Tzermia et al., 1994).

The organic anions α-ketoglutarate, *para*-aminohippurate, folate, urate, and valproate are substrates of members of the **OAT** family. Some of these OATs have homology with members of another subfamily of the MFS, the sugar porter family.

The metabolites transported by members of the **MHS** family have little in common, except that they all possess at least one carboxyl group. The SHS family member JEN1 also has homology with members of this family. Whether MHS members are able to transport monocarboxylated sugars is not known, but appears likely.

The **ACS** family contains bacterial members which transport hexuronates, glucarate or galactonate. The sialic acid transporter is the only mammalian transporter known to transport free hexuronates. Interestingly, the eukaryotic members of this family are sodium/phosphate cotransporters (Pao et al., 1998). Whether the sialic acid transporter is able to transport phosphate in the presence of a sodium gradient has so far not been investigated.

In conclusion, the lysosomal sialic acid transporter belongs to a limited number of anion transporter families. We should, therefore, not limit our search for the sialic acid transporter gene among homologues of the MCT family, but also screen for candidates among the SHS, OAT, MHS, GntP and ACS gene families. The Human Genome Mapping Project has provided a large collection of expressed sequence tags (ESTs) in databases. These databases contain many ESTs which have homology to members of these transporter families, and represent novel transporters with unknown functions. By

mapping these ESTs and comparing their chromosomal localization to the known critical SASD region on chromosome 6q, candidate genes can be selected (Leppänen et al., 1996).

## 2.3 Characterization of a novel lysosomal heavy metal ion transporter

Several heavy metal ions, like copper, zinc, and iron, are required in trace amounts as essential cofactors of many biological processes. The existence of multiple heavy metal transport systems has been demonstrated in both prokaryotes and eukaryotes in order to maintain metal ion homeostasis (Solioz and Vulpe, 1996; Paulsen and Saier, 1997; Eng et al., 1998). Their vital importance is reflected by the occurrence of human genetic disorders which are due to a defect in a putative heavy metal transport protein. Menkes and Wilson disease are both genetic disorders with a disturbed copper metabolism (DiDonato and Sarkar, 1997). Both genes (ATP7A and ATP7B, respectively) encode putative copper transporting P-type ATPases. They are predominantly located in the *trans*-Golgi network where they probably serve as an import protein for copper ions to provide copper to the cuproenzymes. A gene encoding a putative mitochondrial iron transporter is mutated in X-linked sideroblastic anemia and ataxia. This recessive disorder is characterized by hypochromic microcytic erythrocytes and a disturbed iron metabolism (accumulation of iron) in the mitochondria of bone marrow erythrocyte precursors (Allikmets et al., 1999).

Lysosomes also play a role in heavy metal metabolism. They are involved in the degradation of ferritin, an intracellular iron-storage protein (Ringeling et al., 1989; Radisky and Kaplan, 1998). Accumulation of iron-protein complexes in hepatocyte lysosomes is observed in hereditary hemochromatosis, one of the most common recessive inherited disorders (Stal et al., 1990). This disease is characterized by increased intestinal iron absorption and progressive iron overload. The protein product of the gene mutated in this disease, HFE, is co-trafficking with the transferrin receptor and has probably a role in intracellular iron regulation (Gross et al., 1998). Hepatocyte lysosomes are also involved in the biliary excretion of heavy metals. This has been observed during experimental copper and iron overload conditions, in which lysosomes excrete their content, including copper and iron, into the bile (Fig. 7) (LeSage et al., 1986; Gross et al., 1989). Accumulation of copper in lysosomes has been observed in human diseases like Wilson disease, Indian childhood cirrhosis, and endemic Tyrolean infantile cirrhosis (Alt et al., 1990; Adamson et al., 1992). A role for lysosomes in heavy metal metabolism has also been suggested in yeast. Yeast mutants with defective vacuoles, the equivalent of lysosomes, are hypersensitive to copper (Eide et al., 1993). This suggests that the vacuole normally is involved in copper detoxification or export. In all these conditions the metal ions need to cross the lysosomal membrane. Direct evidence for a lysosomal heavy metal ion transporter was however lacking.

ATP7A and ATP7B, defective in Menkes disease and Wilson disease respectively, are members of the CPx-type ATPase family, a subgroup of the extensive family of P-type ATPases, which comprises copper and cadmium ATPases (Solioz and Vulpe, 1996). It has been demonstrated that a member of this family, CopB from *Enterococcus hirae,*

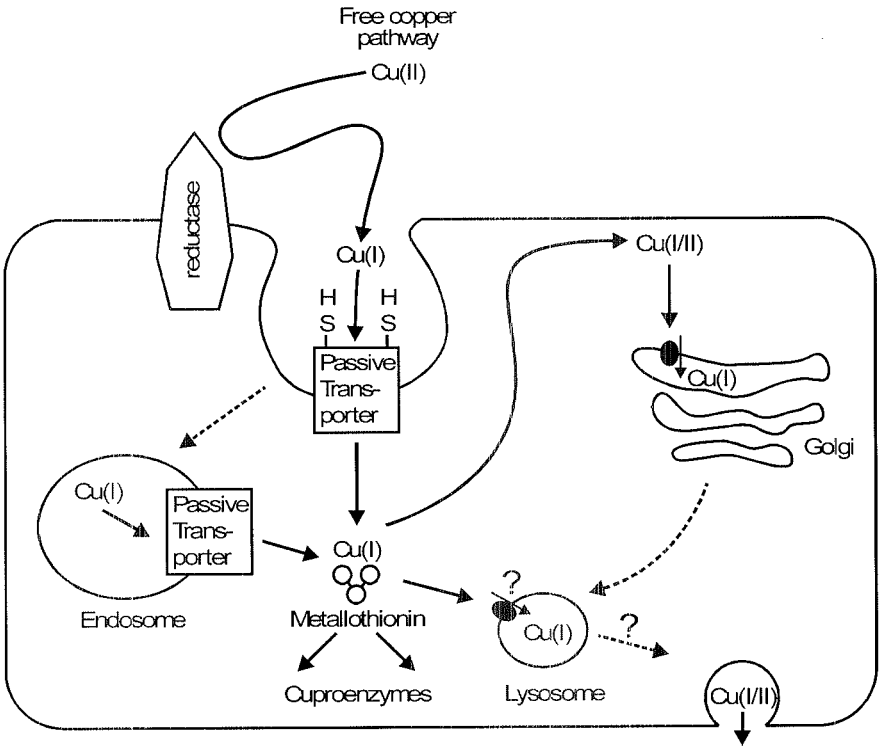


Figure 7. A model for free copper transport in hepatocytes, as adapted and modified from Vulpe and Packman (1995). Cu(II) is reduced by a reductase to Cu(I). Cu(I) enters the cytoplasm via a passive transport protein. Some copper transport into the cytosol occurs at the cell surface, and some occurs after internalization of the copper transporter complex in an endosome. Most free copper will be bound to metallothionein in the cytosol, and will subsequently be transferred to either cytosolic cuproenzymes like superoxide dismutase or to cuproenzymes in the *trans*-Golgi network. ATP7A and ATP7B are probably involved in the translocation of copper across the Golgi membrane. A route for copper from the Golgi to lysosomes is still hypothetical. In the case of hepatic copper overload (like in Wilson disease), cytosolic copper may enter lysosomes, which subsequently excrete their contents into the bile. How copper enters the lysosomes was not understood.

also transports monovalent silver ions, which suggested that copper is transported by CopB as the reduced monovalent cation (CuI) (Solioz and Odermatt, 1995). A fibroblast copper loading test (using $^{64}Cu$) has long been used for the diagnosis of Menkes disease. This test can only be performed in a few specialized centers in the world, because of the

scarce availability and a very short physical half life (12.8 hours) of radioactive copper. In **publication III**, we investigated whether the copper transporter which is defective in Menkes disease, ATP7A, is able to transport silver. It was found that silver ($^{110m}$Ag), indeed can replace $^{64}$Cu and a less cumbersome loading test can be performed for the diagnosis of Menkes disease. Furthermore, it was found that the reduction of divalent to monovalent copper is an essential step preceding transport. $^{110m}$Ag is commercially available and has a convenient physical half life of 250 days. Since CopB and ATP7A, both known as copper P-type ATPases are able to transport monovalent silver, we decided to characterize heavy metal ion transport across the lysosomal membrane, using radioactive silver.

A complication in the studies of metal transport is the aspecific binding of the free metal ions to proteins. *In vivo*, metal ions are bound to special proteins, like ceruloplasmin (copper ions) and transferrin (iron ions). During the transport assays an excess of glutathione was added to bind the free metal ions. In **publication IV**, we have biochemically characterized the first lysosomal heavy metal ion transporter, using intact lysosomes as well as highly purified lysosomal membrane vesicles. The uptake of silver showed the typical kinetics of a (single) carrier-mediated process, which was stimulated by ATP hydrolysis and competitively inhibited by $Ag^+$, $Cu^{2+}$ and $Cd^{2+}$ ions. Biochemically, the lysosomal transporter showed similarities with CopB and ATP7A. Interestingly, the lysosomal transport system does not discriminate between monovalent and divalent ions, since cadmium is only occurring as a divalent ion and silver only as a monovalent ion. Copper, however, can occur as a monovalent or as a divalent ion. The excess of glutathione in the assay probably reduced $Cu^{2+}$ to $Cu^+$. How the transporter translocates both mono and divalent metal ions is not understood yet. We assume that $Ag^+$ (or $Cu^+$) forms a complex with glutathione and that this complex is recognized by the transport protein. Since the lysosomal membrane is impermeable to glutathione, $Ag^+$ is probably released from the complex and transported as a free monovalent ion into the vesicles (Gahl et al., 1985).

Physiologically, this transporter appears to work as an import mechanism for copper and cadmium, since it is stimulated by ATP (the intra-lysosomal lumen does not contain ATP) and transport could be measured into intact lysosomes in the presence of ATP. As an import mechanism, it may explain how copper is taken up into the lysosomes during copper overload conditions. Silver is not known to have any physiological function, although there is sporadic evidence which suggests that silver ions play a role in the oxidative burst of polymorphonuclear leucocytes (Jansson and Harms-Ringdahl, 1993).

In Wilson disease the hepatic copper accumulation is caused by a decreased biliary excretion of this heavy metal. However, the exact (intra)cellular localization of the putative copper transporter is still debated. Long Evans Cinnamon (LEC) rats are the animal model for Wilson disease (Li et al., 1991). Since lysosomes appear to be involved

in the mobilization of copper into the bile during copper overload (Gross et al., 1989), we were interested whether the lysosomal transporter is defective in this disorder. Silver transport in lysosomal membrane vesicles of LEC rats was normal, indicating that the lysosomal heavy metal transporter is different from the ATP7B copper transporter, causing Wilson disease (**publication IV**).

Recently, the *ATP7B* gene has been excluded as a candidate gene for endemic Tyrolean infantile cirrhosis (non-Indian childhood cirrhosis), a rare human copper overload disease resembling Wilson disease (Wijmenga et al., 1998). In this disorder, and probably also in Indian childhood cirrhosis, a genetic predisposition causes rapid copper accumulation upon ingestion of relatively trivial amounts of copper (Muller et al., 1996). It would be interesting to test lysosomal heavy metal transport with radioactive silver in these disorders. However, technical difficulties in obtaining enough material (lysosomal membranes from patient livers) limit at the moment the realization of this test. The *ATP7B* gene, localized on human chromosome 13q14.3, has also been excluded as a candidate gene in the Bedlington terrier, an canine model for copper toxicosis (van de Sluis et al., 1999). The copper toxicosis locus in Bedlington terriers has been mapped to canine chromosome 10, in a region syntenic to human chromosome region 2p13-p16. The phenotype of this animal resembles those of the endemic Tyrolean infantile cirrhosis and Indian childhood cirrhosis, and is therefore suggested as an animal model for these diseases (van de Sluis et al., 1999). Possibly, this canine model can be helpful in the study on lysosomal heavy metal transport in the copper overload disorders.

## Chapter 3 PUBLICATIONS

**Publication I.** **Purification of the lysosomal sialic acid transporter. Functional characteristics of a monocarboxylate transporter.**
(J. Biol. Chem. 1998; 273: 34568-34574)

**Publication II** **Transport of organic anions by the lysosomal sialic acid transporter: a functional approach towards the gene for sialic acid storage disease.**
(FEBS Lett. 1999; 446:65-68)

**Publication III** **Fibroblast silver loading for the diagnosis of Menkes disease.**
(J. Med. Genet. 1998; 35:849-851)

**Publication IV** **Characterization of a heavy metal ion transporter in the lysosomal membrane.**
(FEBS Lett. 1998; 436:223-227)

## PUBLICATION I

**Purification of the lysosomal sialic acid transporter. Functional characteristics of a monocarboxylate transporter**

Havelaar, A.C., Mancini G.M.S., Beerens, C.E.M.T., Souren, R.M.A., and Verheijen, F.W.

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# Purification of the Lysosomal Sialic Acid Transporter

## FUNCTIONAL CHARACTERISTICS OF A MONOCARBOXYLATE TRANSPORTER*

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**Sialic acid and glucuronic acid are monocarboxylated monosaccharides, which are normally present in sugar side chains of glycoproteins, glycolipids, and glycosaminoglycans. After degradation of these compounds in lysosomes, the free monosaccharides are released from the lysosome by a specific membrane transport system. This transport system is deficient in the human hereditary lysosomal sialic acid storage diseases (Salla disease and infantile sialic acid storage disease, OMIM 269920). The lysosomal sialic acid transporter from rat liver has now been purified to apparent homogeneity in a reconstitutively active form by a combination of hydroxyapatite, lectin, and ion exchange chromatography. A 57-kDa protein correlated with transport activity. The transporter recognized structurally different types of acidic monosaccharides, like sialic acid, glucuronic acid, and iduronic acid. Transport of glucuronic acid was inhibited by a number of aliphatic monocarboxylates (*i.e.* lactate, pyruvate, and valproate), substituted monocarboxylates, and several dicarboxylates. *cis*-Inhibition, *trans*-stimulation, and competitive inhibition experiments with radiolabeled glucuronic acid as well as radiolabeled L-lactate demonstrated that L-lactate is transported by the lysosomal sialic acid transporter. L-Lactate transport was proton gradient-dependent, saturable with a $K_m$ of 0.4 mM, and mediated by a single mechanism. These data show striking biochemical and structural similarities of the lysosomal sialic acid transporter with the known monocarboxylate transporters of the plasma membrane (MCT1, MCT2, MCT3, and Mev).**

The major function of lysosomes is the degradation of a large variety of intra- and extracellular macromolecules. The release of degradation products from the lysosome is accomplished by specific membrane transport systems. More than 20 lysosomal transporters have been characterized for specific solutes like amino acids, sugars, nucleosides, ions, and vitamins (1). Their fundamental role in biology is illustrated by the occurrence of two human inherited diseases with a defective lysosomal transport function, cystinosis and sialic acid storage diseases (2). Sialic acid storage diseases are autosomal recessive disorders that are characterized by mental retardation and a variable degree of neurodegeneration. Lysosomal accumulation and excessive urinary excretion of free sialic acid are pathognomonic findings. Previously, we have characterized a carrier in the lysosomal membrane with substrate specificity for the acidic monosaccharides sialic acid (Neu5Ac)[1], uronic acids, and aldonic acids (3). Subsequent studies in our laboratory showed that a defective transport of sialic and glucuronic acid (GlcA) is the primary defect in both clinical variants (4), Salla disease and infantile sialic acid storage disease. Recently, the gene for these disorders has been localized to the same refined chromosomal area on 6q14-q15 by linkage disequilibrium analysis (5). However, the disease gene has not been identified yet. The elucidation of the molecular structure and functional properties of the lysosomal sialic acid transporter is indispensable for further understanding of the molecular defect(s) in the clinical heterogeneous forms of sialic acid storage diseases. Previously, we have developed a functional reconstitution system for the sialic acid transporter that provided the tool to start the purification and functional characterization of the transport protein (6).

In this paper we present the purification of the sialic acid transporter from lysosomal membranes of rat liver to apparent homogeneity. Its functional properties are compared with those of other monocarboxylate transporters present in the plasma membrane of various mammalian cells (7–9).

## EXPERIMENTAL PROCEDURES

### *Materials*

Highly purified lysosomal membrane vesicles were isolated from livers of adult Wistar rats (3). The lysosomal membrane vesicles were suspended at a protein concentration of 8–10 mg/ml in 20 mM NaHepes, pH 7.4, 1 mM EDTA and were stored at −70 °C. All chemicals used were obtained from Sigma or as indicated. L-Iduronic acid, sodium salt was obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). All the tested carboxylates were titrated with NaOH before use.

### *Reconstitution*

Reconstitution of the protein eluates into liposomes was performed as described earlier (6), with the following modification: proteoliposomes were formed by incubating the protein sample, containing detergent and phospholipid (total volume, 170 μl), with 150 μl of Amberlite XAD-2 beads (Fluka) in 20 mM NaHepes, pH 7.4, 100 mM KCl. After 30 min of rotation at room temperature, beads were removed by short centrifugation, and proteoliposomes were used for transport assays.

### *Transport Assay*

After reconstitution, the carrier activity was assayed by uptake of radiolabeled GlcA in the presence of an inwardly directed proton gradient. Because Neu5Ac and GlcA are transported by the same lysosomal transporter for acidic monosaccharides (3, 4), we have performed all studies using radiolabeled GlcA, which was more readily available. Aliquots of proteoliposomes (25 μl) were incubated at 37 °C with 5 μl of 240 mM Mes (free acid) containing 2 μCi of D-[1-$^3$H]GlcA (Amersham Pharmacia Biotech; specific activity, 6.6 Ci/mmol), resulting in an extravesicular pH of 5.5 and a final concentration of 10 μM GlcA. Blank

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; GlcA, glucuronic acid; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; IdoA, iduronic acid. DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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values were determined by incubation of proteoliposomes at 37 °C with 40 mM Mes (free acid), 7 mM unlabeled NaGlcA, pH 5.5, and 2 μCi of D-[1-$^3$H]GlcA and subtracted from all determinations. Previous experiments showed that uptake rates are linear up to 1 min. After 1 min, the reactions were stopped by diluting the sample with 70 μl of ice-cold incubation buffer (17 mM NaHepes, 84 mM KCl, 40 mM Mes (free acid), pH 5.5). The samples were immediately applied to a Sephadex G50 fine (Amersham Pharmacia Biotech) column (Pasteur pipettes, 0.5 × 5cm) at 4 °C. Columns were equilibrated in cold incubation buffer, and vesicles were eluted with 1 ml of cold incubation buffer. Vesicle-associated radioactivity was determined by liquid scintillation counting in 10 ml of Insta-gel (Packard).

*cis*-Inhibition experiments were performed by incubating the proteoliposomes for 1 min at 37 °C with 2 μCi of [$^3$H]GlcA (final concentration, 10 μM) in 40 mM Mes (free acid), resulting in an inwardly directed proton gradient ($pH_{in}$ = 7.4 > $pH_{out}$ = 5.5), and 7 mM of the tested compound.

For *trans*-stimulation studies, a 60% proteoliposome solution (25 μl) was pre-incubated for 60 min at 37 °C with 17 mM NaHepes, 84 mM KCl, 40 mM Mes acid, pH 5.5, plus 10 μM monensin, 10 μM valinomycin (Boehringer Mannheim) in the presence or absence of 1 mM unlabeled substrate. The assay was started by adding 75 μl of an equivalent buffer containing 2 μCi of [$^3$H]GlcA at 37 °C with a final concentration of 0.25 mM. When the samples were pre-incubated without unlabeled substrate, the external final concentration was corrected as in the case of preloading (0.25 mM unlabeled compound). After 1 min, the reaction was stopped as described (6).

The experiments with [$^{14}$C]L-lactate (Amersham Pharmacia Biotech; specific activity, 152 mCi/mmol) were largely performed as described for [$^3$H]GlcA. However, incubation mixtures contained 0.066 μCi of [$^{14}$C]L-lactate (final concentration, 15 μM) and were performed at 20 °C instead of 37 °C. Blank values were determined by incubation of proteoliposomes with 40 mM Mes (free acid), 7 mM unlabeled sodium L-lactate and subtracted from all determinations. For protein side chain modification, proteoliposomes (100 μl) were incubated and treated as described (6).

### *Purification of the Rat Liver Lysosomal Membrane Sialic Acid Transport Protein*

For a single purification, lysosomal membrane vesicles prepared from 150 g of rat livers (15 rats) were used.

*Step 1: Solubilization*—Solubilization of lysosomal membrane proteins was performed by mixing the lysosomal membrane vesicles 1:1 (v/v) with 1% Triton X-100 (especially purified for membrane research, Boehringer Mannheim), 20 mM Tris-HCl, pH 7.4. After 25 min of incubation at 0 °C, unextracted material was pelleted by ultracentrifugation at 150,000 × *g* in a Beckman SW 40 rotor for 20 min at 4 °C.

*Step 2: Hydroxyapatite*—The Triton X-100 extract was applied to hydroxyapatite columns (Pasteur pipettes containing 0.5 g of dry material, Biogel HTP, Bio-Rad, packed by 15 s of tapping) at 4 °C, with a maximum of 500 μl solubilized material/column. Each column was washed with 3 ml of 20 mM Tris-HCl, pH 7.4, 0.1% Triton X-100 (buffer A). Elution was with 3 ml of buffer A, 25 mM $Na_2HPO_4$, $NaH_2PO_4$, pH 7.4. After pooling all the 3-ml eluates, a 2-ml sample was concentrated in a Centricon 10 device (Amicon, Inc., Beverly, MA) until 100–150 μl and desalted. A 50-μl aliquot was used for the reconstitution assay and a 20-μl aliquot was used for the protein assay. Desalting was performed on a 2-ml Sephadex G50 medium (Amersham Pharmacia Biotech) column equilibrated in buffer A (10).

*Step 3: Lentil Lectin*—The eluates of the different hydroxyapatite columns were pooled and applied to a 2-ml lentil lectin affinity chromatography column (lentil lectin-Sepharose 4B, Amersham Pharmacia Biotech) pre-equilibrated in buffer A. After washing the lentil lectin column with 2 ml of buffer A, the flow-through fraction (unretained material) was applied to a 2-ml DEAE-Sephacel (Amersham Pharmacia Biotech) anion exchanger pre-equilibrated in buffer A containing 10% glycerol (buffer B). A 2-ml sample of the lentil lectin flow-through fraction was concentrated in a Centricon 10 device to 100–150 μl and desalted (10). A 50-μl aliquot was used for the reconstitution assay, and a 20-μl aliquot was used for the protein assay.

*Step 4: DEAE-Sephacel*—After extensive washing the DEAE-Sephacel column with 20 ml of buffer B and 20 ml of buffer B with 40 mM NaCl, bound material was eluted with 6 ml of buffer B with 100 mM NaCl. This fraction was stored at −70 °C.

*Step 5: Hydroxyapatite*—After the DEAE-eluate was thawed, a 0.5 ml sample was concentrated in a Centricon 10 device until 100 μl and desalted (10). A 50 μl aliquot was used for the reconstitution assay and a 20 μl aliquot for the protein assay. The 100 mM NaCl DEAE-eluate (5.5 ml) was adjusted to pH 6.0 with 0.5 M Mes (free acid) and applied to a prepacked hydroxyapatite column (1-ml EconoPac HTP cartridge, Bio-Rad) pre-equilibrated in 300 mM NaCl, 20 mM NaMes pH 6.0, 0.1% Triton X-100. Transport activity was eluted with 6 ml of 1 mM $Na_2HPO_4$, $NaH_2PO_4$, pH 6.0, 300 mM NaCl, 0.1% Triton X-100. This eluate was concentrated in Millipore ultrafree-15 centrifugal filters 10K (Millipore Corporation, Bedford) to approximately 300 μl and desalted (10). A 50-μl aliquot was used for the reconstitution assay, and a 100-μl aliquot was used for the protein assay.

*Step 6: Mono Q*—The concentrated hydroxyapatite eluate (150 μl) was applied to a 0.10-ml Mono Q anion exchange column attached to a Amersham Pharmacia Biotech SMART system. This column was equilibrated in buffer B, and bound material was eluted with a linear gradient of 0–210 mM NaCl in buffer B. Fractions of 0.1 ml were collected and pooled pairwise, buffer was exchanged for 20 mM Na-Hepes, 100 mM KCl, 0.1% Triton X-100 by the desalting procedure as described above, and a 50-μl aliquot was used for the reconstitution assay. All column procedures were performed at 4 °C.

### *Protein Characterization and Determination*

The purity of the various active fractions was determined by SDS-polyacrylamide gel electrophoresis according to Laemmli (11) of methanol/chloroform precipitated samples (12), followed by Coomassie Brilliant Blue R-250 or the silver nitrate staining according to Amersham Pharmacia Biotech. Protein concentration was determined by the procedure of Lowry *et al.* as modified by Peterson (13) for the presence of Triton. Protein concentrations in eluates of the second hydroxyapatite column were determined after methanol/chloroform precipitation (12). Protein concentrations in Mono Q eluates were too low to be determined by the above assay and were therefore estimated from silver-stained SDS-PAGE gels.

For the endoglycosidase F/*N*-Glycosidase F (Boehringer Mannheim) treatment of the purified protein, the Mono Q fractions 19–23 were pooled, concentrated, and incubated with 25 milliunits endoglycosidase F, 100 μl in the presence of 20 mM potassium phosphate buffer, pH 7.4, 50 mM EDTA, 2% Triton X-100, 0.2% SDS, 2% β-mercaptoethanol for 2 h at 37 °C. Proteins were precipitated with methanol/chloroform (12). The pellet was resuspended in sample buffer and analyzed by SDS-PAGE (10% gel).

## RESULTS

*Purification of the Lysosomal Sialic Acid Transporter*—Various membrane (transport) proteins have been successfully purified using hydroxyapatite as well as ion exchange chromatography in the presence of detergents (14–18). In addition, affinity chromatography with oligosaccharide-specific lectins has been used to identify the major heavily glycosylated lysosomal membrane proteins: LAMPs (lysosomal-associated membrane proteins) and LIMPs (lysosomal integral membrane proteins) (19–22). Based on the success of these purification methods for membrane proteins we developed a purification protocol for the lysosomal sialic acid transporter. Previously, we have reported a successful reconstitution procedure for the rat liver lysosomal sialic acid transporter that now provided the functional assay to follow fractionation and purification of the solubilized transporter (6). At all steps of the purification procedure, samples were collected and reconstituted into proteoliposomes, and their transport activities were measured using radiolabeled GlcA as a substrate (Table I).

The Triton X-100 solubilized lysosomal membrane proteins were applied to small columns of dry hydroxyapatite material. The columns were washed with equilibration buffer at pH 7.4, and about 20% of the transport activity was eluted with 25 mM sodium phosphate buffer at pH 7.4. This resulted in a 4-fold purification. The next step consisted of lentil lectin affinity chromatography. Almost all activity of the sialic acid transporter was recovered from the column flow-through. Lentil lectin recognizes α-D-glucose and α-D-mannose residues and therefore binds glycoproteins. Consequently, a number of major lysosomal membrane glycoproteins bound to the column and thus could be separated from the protein preparation containing transport activity. This step was kept in our protocol

TABLE I

*Purification of the sialic acid transporter from rat liver lysosomal membrane vesicles*

Lysosomal membrane vesicles (approximately 25 mg of protein) derived from 150 g of rat livers were used as starting material. The purification procedure, reconstitution, and transport assay were performed as described under "Experimental Procedures." Activity is expressed as uptake of [$^3$H]GlcA in 1 min at 37 °C. Data represent the means of three separate isolations.

| Fraction | Protein | Total protein | Total Activity | Yield | Specific activity | Fold enhancement |
|---|---|---|---|---|---|---|
| | *μg/ml* | *μg* | *pmol/min* | % | *pmol GlcA/mg/min* | |
| Solubilized lysosomal membrane extract | 200 | 12000 | 2102.4 | 100 | 175.2 | 1 |
| First hydroxyapatite eluate | 16.4 | 591 | 441.5 | 21 | 747.3 | 4 |
| Lentil lectin eluate | 13.6 | 489 | 378.4 | 18 | 774.4 | 4.4 |
| DEAE eluate | 16.6 | 99.5 | 252.3 | 12 | 2534.9 | 14.5 |
| Second hydroxyapatite eluate | 0.5 | 3.0 | 42.1 | 2 | 14171.4 | 80 |
| Mono Q eluate | 0.14 | 0.028 | 2.1 | 0.1 | 75757.6 | 432 |

despite the fact that it did not lead to an increase in specific activity.

The lentil lectin flow-through fraction was applied to a DEAE-Sephacel anion exchange column. With 100 mM NaCl, 12% of the total transport activity was eluted. As depicted in Table I, this resulted in a ≈14.5-fold increase in specific activity over the starting material. Analysis of the protein composition of fractions obtained from these initial purification steps is shown in Fig. 1. Many different protein bands were still present.

The next purification step consisted of chromatography on hydroxyapatite. This time the column was pre-equilibrated at pH 6.0 in the presence of 300 mM NaCl. Under these conditions, acidic proteins are retained and are eluted with low phosphate buffers. This step provided an important purification of the sialic acid transport protein with an 80-fold enrichment in specific activity (Table I). SDS-PAGE protein analysis using silver staining showed at least four distinct protein bands (Fig. 2*A*). One of these proteins has a molecular mass of 85 kDa and based on its N-terminal amino acid sequence represented one of the major lysosomal membrane glycoproteins, the Lgp85 or LIMP II (23). Another major 67-kDa protein represented the lysosomal membrane-bound subunit of acid phosphatase (24). The other proteins were considered as candidates for the lysosomal sialic acid transporter.

The next purification step consisted of a strong anion exchange Mono Q column attached to the SMART system of Amersham Pharmacia Biotech. Retained proteins were eluted with a gradient of 0–210 mM NaCl. SDS-PAGE analysis by silver nitrate staining of the eluted proteins showed a predominant protein band with a molecular mass of ≈57 kDa in the fractions 20/21 in which also the highest GlcA transport activity was observed (Fig. 2*B*). In addition, quantitative image analysis of the SDS-PAGE protein elution pattern from the Mono Q column demonstrated a correlation between the 57-kDa protein and the transport activity (data not shown). All other visualized proteins could not represent the sialic acid transporter, because they became more prevalent in following fractions, where lower or no transport activity was detected (Fig. 2*B*).

In the final protein preparations (fractions 20–21 from the Mono Q column) transport activity was 432-fold enriched over the activity in the initial lysosomal membrane extract (Table I). Considering that the lysosomal membrane marker β-glucosidase is about 100-fold enriched in the lysosomal membrane vesicles (used as a starting material), the sialic acid transport protein is about 40,000-fold purified in the final eluate of the Mono Q column.

*Properties of the Purified Lysosomal Sialic Acid Transporter*—To investigate the glycosylation of the transporter, the final protein preparation was incubated with the enzyme mixture endoglycosidase F/*N*-glycosidase F. After treatment, the apparent molecular mass of the 57-kDa protein was not decreased. The apparent molecular mass of a control glycoprotein was decreased as a result of cleavage of glycosydic chains (data not shown). This, together with the observation that this protein did not interact with lentil lectin, indicates that the carrier is apparently not glycosylated. Analysis by SDS-PAGE in the presence or absence of the thiol-reducing agent 2-mercaptoethanol did not show any alteration of the electrophoretic behavior of the purified transport protein (data not shown). This indicates that the transporter is not functional as a (homo)dimer or polymer linked by disulfide bridges.

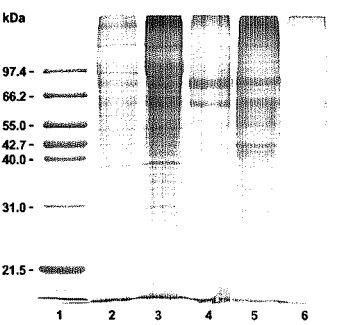


FIG. 1. **SDS-PAGE of protein fractions during the initial steps of the purification of the functional lysosomal sialic acid transporter from rat liver.** Protein fractions were analyzed by SDS-PAGE (10% gel) and Coomassie Brilliant Blue R-250 stained. Per lane, an aliquot of approximately 30 μg of total protein was loaded. *Lane 1*, mid-range protein molecular weight markers (Promega). *Lane 2*, rat liver lysosomal membrane vesicles. *Lane 3*, Triton X-100-solubilized lysosomal membrane vesicles. *Lane 4*, 25 mM sodium phosphate eluate of first hydroxyapatite column. *Lane 5*, lentil lectin unretained fraction. *Lane 6*, 100 mM NaCl eluate of DEAE-Sephacel column.

*Substrate Specificity of the Lysosomal Sialic Acid Transporter*—Because the final yield of the highly purified sialic acid transporter was very low, detailed kinetic studies were difficult to perform. Therefore, most kinetic characterization of the lysosomal sialic acid transporter was performed using partially purified preparations (DEAE-Sephacel eluates). Subsequently, some key experiments were repeated in a concise manner with the highly purified transport preparation.

*Interaction of the Lysosomal Sialic Acid Transporter with Iduronic Acid*—In earlier substrate specificity studies with the crude lysosomal sialic acid transporter, we have shown that this transporter recognizes structurally different types of acidic monosaccharides (*i.e.* the sialic acid Neu5Ac and the uronic acid GlcA) (3, 4). The uronic acid iduronate (IdoA) represents,

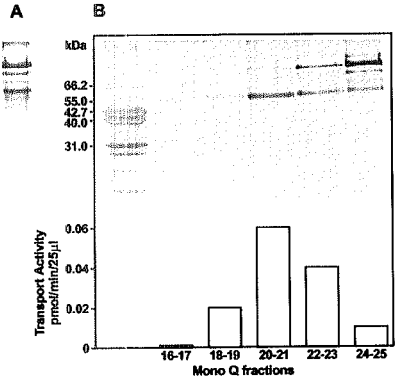


FIG. 2. **A 57-kDa protein correlates with the transport activity.** *Panel A* shows the SDS-PAGE protein pattern of the preparation, which was applied to the Mono Q column. *Panel B, bottom*, elution profile of Mono Q column. Transport activity was measured in reconstituted proteoliposomes of the respective Mono Q fractions. Proteoliposomes (25 $\mu$l) were incubated 1 min at 37 °C with 10 $\mu$M [$^3$H]GlcA in the presence of an inwardly directed proton gradient. Transport activity is expressed as pmol [$^3$H]GlcA/min/25 $\mu$l. *Top*, SDS-PAGE and silver staining of corresponding Mono Q fractions.

like GlcA, a major component of glycosaminoglycans. These are degraded in lysosomes, and thus free IdoA is like GlcA expected to be transported across the lysosomal membrane. The recent commercial availability of free IdoA made it now possible to investigate by *cis*-inhibition and *trans*-stimulation studies whether this uronic acid is also a substrate for the lysosomal sialic acid transporter (Table II). IdoA inhibited [$^3$H]GlcA uptake, although less efficiently than Neu5Ac and GlcA. Furthermore, IdoA was able to induce, like its isomer GlcA, almost a 2-fold *trans*-stimulation (Table II). These experiments indicate that IdoA is indeed a substrate for the sialic acid transporter.

*Interaction of the Lysosomal Sialic Acid Transporter with Small Monocarboxylates*—We investigated the interaction of the transport protein with other known substrates for organic anion carriers. Initially, mono-, di-, and tricarboxylic acids were tested for their *cis*-inhibition effect on the initial linear rate of proton-driven [$^3$H]GlcA uptake in a partially purified preparation (Table II). Most of these organic anions are known substrates for the proton-driven monocarboxylate transporters MCT1, MCT2, and MCT3 of the plasma membrane and for the pyruvate and the dicarboxylate transporters of the outer mitochondrial membrane. The monocarboxylic and dicarboxylic acids were all strong inhibitors, except for the amino acid glutamate and the Krebs cycle intermediate $\alpha$-ketoglutarate. L-Lactate and the anti-epileptic drug valproic acid (dipropyl acetate), among the monocarboxylates, and succinate, among the dicarboxylates, were the strongest inhibitors (Table II). The tricarboxylate citrate showed no significant inhibition. To test whether inhibition represents interaction at the substrate binding site and consequently transport, we investigated the *trans*-stimulation effect of some representative mono- and dicarboxylate inhibitors on the uptake of [$^3$H]GlcA. Partially purified protein preparations were reconstituted in proteoliposomes and preloaded with an unlabeled compound at concentrations of 1 mM, just above the $K_m$ of GlcA (0.4 mM) (6), and the uptake of [$^3$H]GlcA was followed for 1 min. As shown in Table II, L-lactate as well as GlcA itself *trans*-stimulated the uptake of [$^3$H]GlcA. Mevalonate and succinate did not cause *trans*-stimulation. Next, we investigated transport kinetics of L-lactate by the partially purified sialic acid transporter using radiolabeled [$^{14}$C]L-lactate. The presence of an inwardly directed proton gradient ($pH_{in}$ = 7.4 > $pH_{out}$ = 5.5) stimulated initial uptake rates of lactate above equilibrium level (Fig. 3). At the top of overshoot, approximately 2% of external lactate was taken up inside the vesicles. This overshoot phenomenon was abolished in the absence of a proton gradient ($pH_{in}$ = $pH_{out}$ = 7.4), indicating that the transport of lactate is proton gradient-driven, similarly to the transport of the acidic monosaccharides Neu5Ac and GlcA. In these experiments, the use of low concen-

TABLE II
*cis-Inhibition and trans-stimulation of [$^3$H]GlcA uptake by mono-, di-, or tricarboxylic acids*

The partially purified (DEAE-Sephacel eluate) sialic acid transporter was reconstituted, and proteoliposomes were incubated 1 min at 37 °C with 10 $\mu$M [$^3$H]GlcA in the presence of an inwardly directed proton gradient and 7 mM of the indicated compounds. Data represent the means of four independent determinations ± S.D. In *trans*-stimulation experiments partially purified proteoliposomes were preincubated for 60 min at 37 °C in the presence or absence of 1 mM unlabeled GlcA, IdoA, L-lactate, mevalonate, or succinate in 20 mM NaHepes, 100 mM KCl, 40 mM Mes, pH 5.5, 10 $\mu$M valinomycin and monensin. The transport assay was started by a 4-fold dilution in pH 5.5 incubation buffer with 2 $\mu$Ci of [$^3$H]GlcA and allowed to proceed for 1 min. In the samples that were preincubated without unlabeled compound, 0.25 mM unlabeled compound was added together with radiolabeled substrate to give the same extravesicular substrate concentration in both experiments.

| | Transport activity | | trans-Stimulation |
|---|---|---|---|
| | *pmol/mg/min* | *% of control* | *% of not trans-stimulated* |
| Control | 2280.5 ± 132.6 | | |
| Acidic monosaccharides | | | |
| GlcA | 0 | 0 | 200 |
| Neu5Ac | 0 | 0 | |
| IdoA | 916.1 ± 272.4 | 40 | 180 |
| Monocarboxylates | | | |
| Oxamate | 553.0 ± 45.5 | 24 | |
| Pyruvate | 534.6 ± 46.0 | 24 | |
| L-Lactate | 195.6 ± 67.8 | 8 | 150 |
| 4-OH-butyrate | 369.4 ± 107.2 | 16 | |
| Mevalonate | 510.0 ± 24.0 | 22 | 109 |
| Valproate | 0 | 0 | |
| Dicarboxylates | | | |
| Succinate | 0 | 0 | 79 |
| Malate | 366.0 ± 21.8 | 16 | |
| Malonate | 685.3 ± 37.5 | 30 | |
| Maleate | 592.5 ± 79.5 | 26 | |
| Fumarate | 234.3 ± 81.0 | 10 | |
| $\alpha$-Ketoglutarate | 1090.2 ± 199.6 | 48 | |
| Glutamate | 2437.8 ± 195.4 | 107 | |
| Tricarboxylate | | | |
| Citrate | 1665.9 ± 30.6 | 73 | |

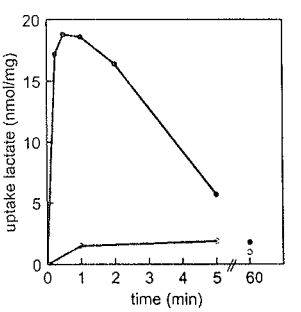


FIG. 3. **Proton gradient-dependent uptake of [$^{14}$C]L-lactate.** Proteoliposomes of DEAE-Sephacel eluate prepared in 20 mM NaHepes, 100 mM KCl, pH 7.4, were incubated with 15 $\mu$M [$^{14}$C]L-lactate at 20 °C in 40 mM Mes (free acid), 10 $\mu$M valinomycin, pH 5.5 (with proton gradient, $pH_{in}$ = 7.4 > $pH_{out}$ = 5.5, ●) or in 20 mM NaHepes, 10 $\mu$M valinomycin, pH 7.4 (no proton gradient, $pH_{in}$ = $pH_{out}$ = 7.4, ○).

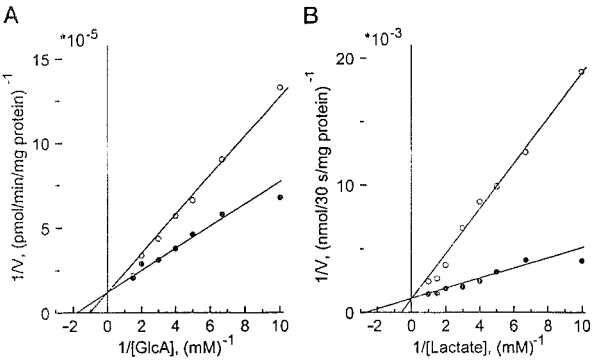


FIG. 4. **Competitive inhibition of [$^3$H]GlcA transport by L-lactate and of [$^{14}$C]L-lactate transport by Neu5Ac.** Initial proton-dependent transport rates of [$^3$H]GlcA (1 min, 37 °C) and [$^{14}$C]L-lactate (30 s, 20 °C) were measured in proteoliposomes of DEAE-Sephacel eluates. The uptake medium contained increasing concentrations of the respective substrates in the presence or absence of the inhibitors L-lactate or Neu5Ac. Data were plotted double reciprocally. *A*, [$^3$H]GlcA uptake with (○) or without (●) cold 2 mM L-lactate. *B*, [$^{14}$C]L-lactate uptake with (○) or without (●) cold 7 mM Neu5Ac.

trations of lactate (15 $\mu$M), lower temperature (20 °C), and the use of a pH far above the pK for the tested compounds limit the contribution of aspecific diffusion on the net uptake. Proton-driven [$^{14}$C]L-lactate transport under apparent zero-*trans* conditions was saturable with a $K_m$ of approximately 0.4 mM and a $V_{max}$ of 500 nmol/30 s/mg protein. An Eadie-Hofstee plot of the kinetic data indicated a linear process, suggesting that only one type of transport system operates (data not shown).

Although these studies provide evidence that the partially purified protein preparation is able to transport, in addition to acidic monosaccharides, many other small monocarboxylic acids, it cannot be excluded that other proteins in this preparation are present. However, the exchange of GlcA with L-lactate in the *trans*-stimulation experiments is strong evidence for transport of both compounds by the same protein. To provide further evidence that lactate and GlcA can be transported by the same carrier in the lysosomal membrane, competitive inhibition experiments were performed. Proton-dependent transport of [$^3$H]GlcA or [$^{14}$C]L-lactate was measured in voltage clamped membranes with $K^+$/valinomycin in the absence or presence of cold L-lactate or cold Neu5Ac as inhibitors, respectively. The results were fitted to a double reciprocal plot, showing a clear mode of competitive inhibition of lactate (calculated $K_i$ of 2.5 mM) on GlcA transport and of Neu5Ac (calculated $K_i$ of 2 mM) on L-lactate transport (Fig. 4). Definite evidence that transport of lactate is performed by the lysosomal sialic acid transporter was obtained from *cis*-inhibition and concentration-dependent inhibition studies with the highly purified sialic acid transporter preparation. Proton-driven [$^3$H]GlcA transport under apparent zero-*trans* conditions was completely inhibited in the presence of 7 mM unlabeled GlcA or Neu5Ac or L-lactate (Table III). Proton-driven [$^{14}$C]L-lactate transport under apparent zero-*trans* conditions was inhibited totally by L-lactate and significantly by Neu5Ac. It is interesting to note that under these conditions GlcA did not inhibit. Because the GlcA transport assays are performed at 37 °C and the lactate transport assays at 20 °C, these apparent inconsistencies can be explained by differences in affinities at different temperatures. Inhibition of [$^3$H]GlcA transport by L-lactate was a clear concentration-dependent process (Table III). Clearly, the highly purified transporter preparation contains a transporter that carries all three substrates, GlcA, Neu5Ac, and L-lactate (see also the above competitive inhibition experiments).

*Sensitivity to Covalent Protein Modifiers*—In previous experiments studying the effect of protein modifiers on GlcA transport in native lysosomal membrane vesicles and reconstituted proteoliposomes, we have demonstrated the involvement of arginines (and possibly histidines) in substrate recognition (3, 6). To investigate whether L-lactate transport is similarly affected by some of these protein modifiers, inhibition and substrate protection experiments were performed. 1 mM *N*-ethylmaleimide, a thiol-modifier, irreversibly inactivated transport of both GlcA (75% inhibition) and L-lactate (86% inhibition) in proteoliposomes from partially purified preparations. Phenylglyoxal, under these conditions an arginine modifier, significantly inhibited GlcA transport (44% of uninhibited rate). The inactivation of GlcA transport could be partially rescued when phenylglyoxal treatment was performed in the presence of the substrates GlcA, Neu5Ac, and L-lactate (96, 96 and 56% of uninhibited rate, respectively). Similarly, L-lactate transport was significantly inhibited by phenylglyoxal (59% of uninhibited rate) and protected in the presence of L-lactate, GlcA, and Neu5Ac (91, 88, and 71%, respectively, of uninhibited rate). Apparently, GlcA, Neu5Ac, and L-lactate all use the same substrate-binding site of the transport protein.

TABLE III

*cis-Inhibition of [$^3$H]GlcA and [$^{14}$C]L-lactate uptake by GlcA, Neu5Ac, and L-lactate in proteoliposomes of the highly purified lysosomal sialic acid transporter*

In the upper part of the table, the Mono Q eluate was reconstituted, and proteoliposomes were either incubated 1 min at 37 °C in the case of [$^3$H]GlcA assay or 30 s at 20 °C in the case of [rosup;14C]L-lactate assay, both in the presence of an inwardly directed proton gradient and with 7 mM of the indicated compounds. In the lower part of the table, proteoliposomes of the highly purified sialic acid transporter were incubated with 10 $\mu$M [$^3$H]GlcA for 1 min at 37 °C in the presence of an inward directed proton gradient ($pH_{in}$ = 7 4, $pH_{out}$ = 5.5) and with various concentrations of L-lactate. Data represent the means of two independent determinations.

| Compound | Transport activity | | | |
|---|---|---|---|---|
| | [$^3$H]GlcA | | [$^{14}$C]L-Lactate | |
| | *pmol/min/assay* | *% of control* | *pmol/30 s/assay* | *% of control* |
| Control | 0.082 | | 15.4 | |
| GlcA | 0 | 0 | 16.2 | 105 |
| Neu5Ac | 0 | 0 | 8.8 | 57 |
| L-Lactate | 0 | 0 | 0 | 0 |
| L-Lactate | | | | |
| 10 $\mu$M | 0.082 | 100 | | |
| 20 $\mu$M | 0.075 | 92 | | |
| 50 $\mu$M | 0.066 | 81 | | |
| 150 $\mu$M | 0.059 | 72 | | |
| 500 $\mu$M | 0.010 | 12 | | |

## DISCUSSION

In this paper we describe the purification of the lysosomal sialic acid transport protein to apparent homogeneity based on its biological activity. To our knowledge, this is the first report

about the purification and detailed kinetic characterization of a lysosomal membrane carrier protein. Initial attempts using specific inhibitors like α-cyanocinnamate as ligands for affinity chromatography have not been successful.[2] Classical chromatographic techniques for membrane proteins were used. Hydroxyapatite chromatography was particularly successful in obtaining fractions enriched in transport activity. This medium has also been successful in the purification of several mitochondrial membrane transport proteins (14). Like some of those carriers, our transporter adsorbed weakly to hydroxyapatite (elution with 25 mM and 1 mM sodium phosphate buffer). This is probably due to a large micellar shell around the protein formed by Triton X-100 molecules. Silver-stained SDS-PAGE gels showed only one protein band with a molecular mass of approximately 57 kDa, correlating with the transport activity of GlcA (Fig. 2*B*). Concerning the enrichment of this lysosomal membrane transporter and the highly sensitive silver staining, it is unlikely that the activity resides in another minor, not visualized protein. Apparently, the more abundant and well characterized integral lysosomal membrane proteins, such as LAMPs and LIMPs, are densely *N*-glycosylated glycoproteins (22). Based on the deglycosylation studies, the sialic acid transporter appears *not* to be heavily glycosylated. For several known anion carriers *N*-glycosylation is not required for their transport function (25). The same is true for some other membrane transporters (25, 27). Some sugar carriers are known to be functional because homodimers possibly linked by S–S bridges (28, 29). In contrast, our transporter does not have a quaternary structure in which subunits are held together by S–S bridges.

In previous studies, we showed that the structural requirements for recognition of the substrate by the carrier were different from those of other proteins involved in sialic acid metabolism (lysosomal sialidase, the Golgi system transporter for CMP-sialic acid and sialyltransferases). For the carrier, the C-1 carboxylic group had to be unsubstituted as well as the native hydroxyl group at C-2 (3). In addition, the kinetic properties of the lysosomal sialic acid transporter were similar to those of other known proton cotransporters for other organic anions (3, 6). Structurally different monosaccharides, such as sialic acid (*N*-acetylneuraminic acid) (a 9-carbon *N*-substituted monosaccharide), glucuronic acid (a hemiacetal-forming acid monosaccharide with a C-6 carboxylic group), and gluconic acid (a non-hemiacetal-forming acid monosaccharide with a C-1 carboxylic group) are all recognized by this carrier (3, 6). In this paper we extended the group of acidic monosaccharide substrates with IdoA, like GlcA an important physiological component of mammalian glycosaminoglycans. The lysosomal sialic acid transporter is the only mammalian transporter known to allow transport of uronic acids, aldonic acids, and *N*-substituted neuraminic acids (3, 4). The physiological role of sialic acid and glucuronic acid transport has earlier been demonstrated by the accumulation of these monosaccharides and by their defective transport in lysosomes of patients with sialic acid storage diseases (4, 30). So far, lysosomal accumulation of IdoA in these patients has not been investigated. The contribution of the different acidic monosaccharides to the pathology remains therefore elusive.

Because aliphatic monocarboxylates like pyruvate inhibit transport (3), we compared the biochemical characteristics of the purified carrier with those of the aliphatic monocarboxylate transporters. To date, several monocarboxylate transporters present in the plasma membrane of various mammalian cells have been cloned and characterized: MCT1, MCT2, and MCT3 (7–9, 31). They are proton-coupled transporters with a broad specificity for short chain monocarboxylates (including lactate and pyruvate), showing differences in cellular distribution. A number of non-sugar mono- and dicarboxylic acids were effective inhibitors of initial uptake rates of GlcA transport by the partially purified lysosomal sialic acid transporter (Table II). The inhibition observed was not dependent on changes in membrane potential, because experiments were performed in the presence of $K^+$ and valinomycin. These monocarboxylates are transported by the MCTs with different affinities, except mevalonate (7). Mevalonate is transported by Mev, a homologue of MCT1 from Chinese hamster ovary cells (32), but is not transported efficiently by MCT1 (8). *trans*-Stimulation experiments demonstrated that mevalonate is not transported by the lysosomal sialic acid transporter (Table II). Most of the tested dicarboxylates are known to be substrates of the dicarboxylate carrier in mitochondria (*e.g.* succinate, malate, and malonate) (33). For the di- and tricarboxylic acids glutamate, α-ketoglutarate, and citrate specific mitochondrial transport proteins have been identified (34). The lack of *trans*-stimulation in our experiments suggested that one of the strongest dicarboxylate inhibitors (*i.e.* succinate) is not transported by the lysosomal sialic acid transporter. Similarly, a lactate transporter in placental brush border membrane vesicles is unable to transport the *cis*-inhibitor succinate (35).

Transport of [$^3$H]GlcA by the partially purified lysosomal sialic acid transporter is *cis*-inhibited and *trans*-stimulated by the small aliphatic monocarboxylate L-lactate. This inhibition of [$^3$H]GlcA transport by L-lactate showed a competitive mode. The same *cis*-inhibition of [$^3$H]GlcA transport by L-lactate was observed using the highly purified preparation, containing one major protein band on SDS-PAGE. This inhibition was clearly dependent on the concentration of the inhibitor L-lactate. Transport of [$^{14}$C]L-lactate by the partially purified lysosomal sialic acid transporter preparation is a proton gradient-mediated process that can be competitively inhibited by Neu5Ac but surprisingly not by GlcA. [$^{14}$C]L-lactate transport by the highly purified preparation also showed clear inhibition by Neu5Ac but not by GlcA. Apparently, at 37 °C ([$^3$H]GlcA transport assay) as well as GlcA, Neu5Ac, and L-lactate are recognized by the transporter, but at 20 °C ([$^{14}$C]L-lactate transport assay) the affinity for L-lactate and Neu5Ac is much higher than for GlcA. This can be explained by structural differences among these molecules, especially at the site of the carboxyl group. Another possible explanation would be the presence of different lactate transporters, one of which is not fully inhibited by the other two compounds. However, kinetic analysis (linear Eadie-Hofstee plots) did not show any evidence for different lactate transporters. In conclusion, all these data provide strong evidence that the lysosomal sialic acid transporter is able to transport besides acidic monosaccharides also other short chain monocarboxylates like L-lactate. The competitive inhibition experiments, the *trans*-stimulation experiments, and the *cis*-inhibition experiments using the highly purified preparation exclude the possibility that the observed L-lactate transport is due to a contaminating protein.

We do not know whether L-lactate is a physiological substrate in the lysosome. However, a probenecid-inhibitable organic anion transporter, possibly also recognizing lactate, was postulated in endosomes from macrophages (36). Probenecid is also an inhibitor of the sialic acid transporter in native lysosomal membranes.[3] Proton cotransport of L-lactate has been described for MCT1, MCT2, and MCT3 by kinetic analysis and by inhibition with *para*-chloromercuribenzoic acid (8, 9, 31). Lac-

[2] G. M. S. Mancini, unpublished results.

[3] G. M. S. Mancini and F. W. Verheijen, personal observation.

tate and pyruvate show a structural similarity with Neu5Ac, of which pyruvate is the natural precursor. Although it would be interesting to investigate whether MCT1 and/or MCT2 also could transport monocarboxylic monosaccharides, it is assumed that MCT1 transports mainly unbranched aliphatic monocarboxylates from C-2 to C-5 but not mevalonate. [$^3$H]GlcA uptake is *cis*-inhibited by the anti-epileptic drug valproic acid. Valproic acid is also a known inhibitor of the mitochondrial pyruvate carrier (37) and of MCT1 in Chinese hamster ovary cells (38), suggesting a similarity between the lysosomal system here investigated and these monocarboxylate carriers. It would be interesting to study in more detail the role of the lysosomal sialic acid transporter in the transport of valproic acid, its metabolites, and other organic anions by future experiments using their radioactive forms.

Both the lysosomal sialic acid transporter and MCTs are sensitive to hydroxycinnamic acid derivatives, the stilbene disulfonate DIDS, and *para*-chloromercuribenzoic acid (6–9). However, the difference in sensitivity to the amino acid modifier *N*-ethylmaleimide distinguishes MCT1 from the lysosomal sialic acid transporter. MCT1 is only poorly inhibited by *N*-ethylmaleimide (7) in contrast to the sialic acid transporter, which is strongly inhibited by *N*-ethylmaleimide (this paper). Another difference between the sialic acid transporter and MCT1 is the lower $K_m$ for lactate transport, 0.4 mM (this paper) *versus* 8 mM for MCT1 (9). Recently, Price *et al.* have cloned and sequenced four new human MCT homologues, MCT4 to MCT7 (39). Their similarities and some strongly conserved sequence motifs provide evidence for an ancient family of transporters. Considering that the conversion of Phe$^{360}$ into Cys changes MCT1 from a lactate transporter to a mevalonate transporter (8, 32), it is possible that minimal structural differences among MCTs account for major differences in substrate specificity. The amino acid modifier phenylglyoxal inactivated the reconstituted sialic acid transporter. This reagent revealed essential arginine/histidine residues at the substrate-binding site of the transporter, because its inactivation could be prevented by concomitant incubation with Neu5Ac or GlcA (6). L-Lactate acts on the same substrate-binding site as GlcA, because the inhibitory effect of phenylglyoxal on the uptake of [$^{14}$C]L-lactate could be prevented by concomitant incubation with L-lactate or GlcA (this paper). Apparently, phenylglyoxal modifies essential arginine residues present in the lactate-binding site of the carrier protein. Also, for the MCTs an essential function for a conserved Arg residue has been proposed (39). In conclusion, we have observed an overlap in substrate specificity for monocarboxylates, a similar proton cotransport mechanism (with consequently the sensitivity to *para*-chloromercuribenzoic acid), a similar sensitivity to hydroxycinnamic acid derivatives and stilbene disulfonates, and the presence of essential arginine residues in the substrate binding sites of the sialic acid carrier and the monocarboxylate carriers. Altogether, these data suggest functional and structural similarities of our transporter with previously characterized monocarboxylate transporters.

The lysosomal transport mechanism for sialic acid is defective in different clinical variants of the neurodegenerative disorder sialic acid storage disease. We demonstrated that the purified sialic acid transporter interacts with lactate, an important energy source in brain. Further studies of lactate metabolism in brain from these patients might help to understand events of neurodegeneration in this disorder. By NMR spectroscopy, it is possible to show abnormalities in metabolites in brain (40). We are not aware of NMR spectroscopy studies in sialic acid storage diseases, which could be applied to study lactate metabolism in brain of these patients.

By N-terminal sequencing, we have obtained a stretch of amino acids from the 57-kDa protein, but still too many doubtful residues are present, making gene cloning difficult. However, the functional and possible structural homology with monocarboxylate transporters may provide a tool to identify and clone the sialic acid transporter gene by screening for homologues in available sequence libraries.

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## PUBLICATION II

**Transport of organic anions by the lysosomal sialic acid transporter: a functional approach towards the gene for sialic acid storage disease.**

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# Transport of organic anions by the lysosomal sialic acid transporter: a functional approach towards the gene for sialic acid storage disease

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**Abstract Transport of sialic acid through the lysosomal membrane is defective in the human sialic acid storage disease. The mammalian sialic acid carrier has a wide substrate specificity for acidic monosaccharides. Recently, we showed that also non-sugar monocarboxylates like L-lactate are substrates for the carrier. Here we report that other organic anions, which are substrates for carriers belonging to several anion transporter families, are recognized by the sialic acid transporter. Hence, the mammalian system reveals once more novel aspects of solute transport, including sugars and a wide array of non-sugar compounds, apparently unique to this system. These data suggest that the search for the sialic acid storage disease gene can be initiated by a functional selection of genes from a limited number of anion transporter families. Among these, candidates will be identified by mapping to the known sialic acid storage disease locus.**



*Key words:* Lysosomal transporter; Sialic acid; Organic anion; Major facilitator superfamily; Salla disease

## 1. Introduction

Recent work has led to the characterization of specific transport systems for monosaccharides in the mammalian lysosomal membrane [1,2]. One of these, the sialic acid transporter has an essential metabolic function in the disposal of acid sugars from the lysosomal compartment after degradation of glycoproteins, glycosaminoglycans and glycolipids. In the human genetic disorders Salla disease and infantile sialic acid storage disease (SASD) (OMIM 269920), the function of this transporter is impaired and a progressive accumulation of acid sugars occurs in the lysosomal compartment [3,4]. The responsible SASD gene(s) is (are) not known but linkage was demonstrated for both phenotypes to chromosome 6q14-q15 [5]. The lysosomal transporter from human fibroblasts and rat liver recognizes monocarboxylic anionic sugars (e.g. sialic acid, glucuronic acid and iduronic acid) and other aliphatic monocarboxylated anions (e.g. L-lactate), showing functional similarities with the previously characterized family of monocarboxylate transporters (MCTs) [1,4,6].

Membrane transporters have been classified into distinct families on the basis of sequence similarities [7]. Within each family, most proteins have a similar substrate specificity, indicating that substrate specificity frequently correlates with phylogeny. In this way several different anion transporter families have been characterized [8]. The MCT family seems structurally and phylogenetically distinct from other families of organic anion transporters. So far, the molecular structure of the lysosomal sialic acid transporter is not known, but initial functional characterization showed some similarities with members of the different anion transporter families [6]. Therefore, in this paper we have compared in more detail the functional properties of the lysosomal sialic acid transporter with those of carriers from different families of anion transporters. Our final aim is to identify and clone the sialic acid transporter gene causing SASD, which can be initiated by this functional approach.

## 2. Materials and methods

### 2.1. Materials

Rat liver lysosomes were isolated by differential centrifugation, and highly purified membrane vesicles were prepared as described [1]. The lysosomal membrane vesicles were suspended at a protein concentration of 8–10 mg/ml in 20 mM NaHEPES, 0.1 mM EDTA at pH 7.4 and were stored at −70°C. [$^3$H]GlcA (specific activity 6.6 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Most chemicals were purchased from Sigma or as indicated.

### 2.2. Transport assays

For a 'zero-trans' uptake assay, lysosomal membrane vesicles were rapidly thawed at 37°C and pre-equilibrated for 10 min in 20 mM NaHEPES, 10 mM KCl and 10 μM valinomycin at 20°C. All uptake studies were performed for 30 s at 20°C and in the presence of an inward-directed proton gradient ($pH_{out} = 5.5 < pH_{in} = 7.4$) as described earlier [4]. For *cis*-inhibition studies 10 μl pre-equilibrated vesicles were incubated with 10 μl of substrate solution containing 0.5 μCi of radiolabelled GlcA (final concentration 2.5 μM) in 20 mM NaHEPES, 80 mM Mes(free acid), resulting in a extravesicular pH of 5.5 and 10 μl of 21 mM of several organic anions (final concentration 7 mM), titrated with NaOH to pH 5.5. The following organic anions were used: α-ketoglutarate, *p*-aminohippurate, urate, salicylate, valproate, methotrexate, folate, glutamate, tetraethylammonium, phosphate, sulfate, D-glucosamine-2-sulfate, D-galactose-6-sulfate, D-glucose-6-phosphate, *N*-acetyl-glucosamine-1-phosphate. The blank value was determined by incubation of vesicles with 7 mM unlabelled GlcA and substracted from all determinations. Incubations were stopped by the addition of 70 μl of ice-cold stop-buffer (13 mM NaHEPES, 27 mM Mes(free acid) and 10 mM KCl, pH 5.5) and 100 μl was immediately applied to a Sephadex G50 fine (Pharmacia Biotech) column (Pasteur pipettes, 0.5×5 cm), equilibrated in cold stop-buffer at 4°C. Vesicles were eluted with 1 ml ice-cold stop-buffer. Vesicle-associated radioactivity was determined by liquid scintillation counting in 10 ml Instagel (Packard).

*Trans*-stimulation of [$^3$H]GlcA uptake was studied at $pH_{in} = pH_{out} = 5.5$ in the presence of the ionophore monensin. 15 μl of vesicles were pre-equilibrated 60 min at 20°C with 40 mM Mes(free acid), 12 mM NaHEPES, 4 mM KHEPES, 1 mM NaGlcA or other organic anions, 10 μM valinomycin and 10 μM monensin (final concentrations). The assay was started by adding 75 μl of an equivalent

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*Abbreviations:* GlcA, glucuronic acid; Neu5Ac, *N*-acetylneuraminic acid, sialic acid; PAH, *p*-aminohippurate; TEA, tetraethylammonium; $GlcNH_2$-2-S, D-glucosamine-2-sulfate; Gal-6-S, D-galactose-6-sulfate; Glu-6-P, D-glucose-6-phosphate; GlcNAc-1-P, *N*-acetylglucosamine-1-phosphate


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buffer containing 2 μCi of radiolabelled GlcA (final concentration 3 μM). Control experiments were performed by pre-equilibration of the membranes with the same buffer without Na-GlcA or other organic anions. To give the same extravesicular substrate concentration in both experiments, 0.25 mM unlabelled organic anion was added together with radiolabelled substrate at the start of the assay.

For competitive inhibition studies, initial proton dependent transport rates (30 s, 20°C) of 10 μM [$^3$H]GlcA were measured at increasing unlabelled GlcA concentrations (0.05, 0.1, 0.2, 0.3, 0.5 and 1 mM final concentrations) in the presence or absence of different inhibitors. Details about the used concentrations of inhibitors are described in the legend. All experiments were performed in duplicate or triplicate.

## 3. Results

In earlier substrate specificity studies, we have shown that the lysosomal sialic acid transporter recognizes structurally different types of organic anions, like monocarboxylic (aldonic, hexuronic and *N*-substituted anionic) sugars and aliphatic monocarboxylates [1,6]. Recent genome sequencing data and a wealth of biochemical and molecular genetic investigations have revealed the occurrence of many families of primary and secondary transporters [7,8]. Since in the lysosomal system a proton gradient provides the driving force for secondary active transport [1], transporter families in which organic anions are known to be transported by a proton symport mechanism are of special interest. In this paper, we investigated which other organic anions are recognized by the lysosomal sialic acid transporter. We tested a variety of typical substrates of previously identified members of the different families which transport organic anions. As representatives of the novel multispecific organic anion transporters (OATs) we tested *p*-aminohippurate (PAH), α-ketoglutarate, urate, salicylate, methotrexate, folate and valproate [9,10]. The dicarboxylate α-ketoglutarate is also a substrate of the α-ketoglutarate:$H^+$ symport permease of *Escherichia coli*, which belongs to a different family [8]. Additionally, we tested substrates of the mammalian inorganic anion transporters of the sulfate permease (SulP) and phosphate:$H^+$ symporter (PHS) family (i.e. sulfate and phosphate) [11–13], of the acidic amino acid transporter of the proton-dependent oligopeptide transporter (POT) family (i.e. glutamate) [8,11], of the organic cation transporters (OCTs) (i.e. tetraethylammonium) [14] and of the bacterial hexose phosphate transporters of the organophosphate:$P_i$ antiporter (OPA) family (i.e. several sugar-phosphates) [15]. As shown in Fig. 1, the lysosomal sialic acid transporter is strongly *cis*-inhibited by the dicarboxylate α-ketoglutarate, the prototype substrate of organic anion transporters PAH, the purine metabolism endproduct urate and the acidic monocarboxylated drug salicylate. Moderate inhibition (25–60% of control) was shown by the anti-epileptic drug valproate and the anionic drugs methotrexate and folate. No significant inhibition was found for the acidic amino acid glutamate, the organic cation tetraethylammonium (TEA), the inorganic anions phosphate and sulfate and sulfated or phosphorylated sugars. This demonstrates that the lysosomal sialic acid transporter recognizes different organic anions, but not acidic amino acids, organic cations, inorganic anions or sugar-phosphates.

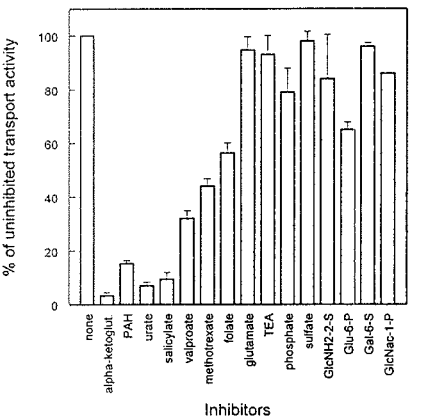


Fig. 1. *Cis*-inhibition of [$^3$H]GlcA uptake into lysosomal membrane vesicles by different anions. Lysosomal membrane vesicles were pre-equilibrated for 10 min, 20°C in 20 mM NaHEPES, 10 mM KCl and 10 μM valinomycin. A sample of 10 μl pre-equilibrated vesicles were incubated for 30 s at 20°C with 2.5 μM [$^3$H]GlcA in the presence of an inward-directed proton gradient and 7 mM of the indicated compounds. The blank value was substracted from all determinations. Data are presented as percentage of the uninhibited transport activity. Values ($n = 3$) are mean ± S.D.

In order to test which of the *cis*-inhibitors can be transported by the lysosomal sialic acid transporter, we investigated their *trans*-stimulation effect on the uptake of [$^3$H]GlcA. For these experiments, vesicles were pre-loaded with unlabelled compound and the uptake of [$^3$H]GlcA was measured in the absence of a proton gradient. In our experience, significant *trans*-stimulation represents at least a two-fold increase above the basal uptake rate under the current condi-

Table 1
*Trans*-stimulation of GlcA uptake by different anions

| anionic compound | Transport Activity | | |
|---|---|---|---|
| | +Preloading | −Preloading | *Trans*-stimulation factor |
| | (pmol/mg/30 s) | | |
| GlcA | 6.53 ± 0.28 | 0.99 ± 0.04 | 6.6 |
| Neu5Ac | 5.90 ± 0.07 | 0.84 ± 0.07 | 7.0 |
| α-Ketoglutarate | 4.35 ± 0.03 | 0.88 ± 0.07 | 4.9 |
| PAH | 2.53 ± 0.17 | 1.56 ± 0.03 | 1.6 |
| Salicylate | 1.87 ± 0.06 | 1.05 ± 0.16 | 1.8 |
| Valproate | 1.84 ± 0.09 | 1.16 ± 0.03 | 1.6 |
| Methotrexate | 1.94 ± 0.17 | 1.62 ± 0.05 | 1.2 |
| Urate | 2.09 ± 0.14 | 1.57 ± 0.07 | 1.3 |
| Glutamate | 1.74 ± 0.05 | 1.32 ± 0.17 | 1.3 |

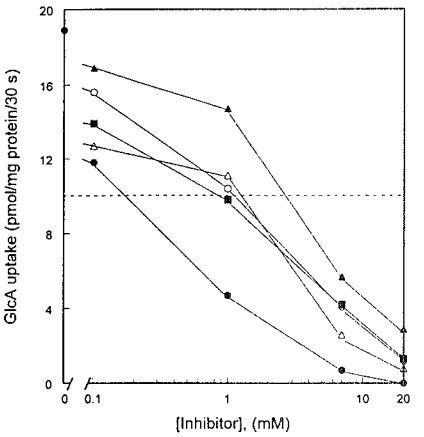


Fig. 2. Concentration dependent inhibition of [$^3$H]GlcA uptake. Transport of 10 μM [$^3$H]GlcA was measured in the presence of α-ketoglutarate (●), valproate (○), PAH (■), folate (▲) and urate (Δ) at the following concentrations: 100 μM, 1, 7 and 20 mM (plotted on a logarithmic x-axis). Transport assays ($n = 3$) were performed as described in the legend of Fig. 1. The blank value was substracted from all determinations. The dotted line corresponds with 50% of the uninhibited rate. The uninhibited rate is indicated on the y-axis (●).

tions. As shown in Table 1, α-ketoglutarate clearly *trans*-stimulated GlcA uptake, like GlcA itself and Neu5Ac. PAH, salicylate, valproate, methotrexate, urate and glutamate did not show a significant *trans*-stimulation at the tested concentrations. This indicates that the lysosomal sialic acid transporter can exchange GlcA for α-ketoglutarate.

Further kinetic studies were performed to determine the mode of inhibition. As shown in Fig. 2, all compounds inhibited in a concentration dependent manner. In subsequent kinetic inhibition studies the following concentrations, close to $IC_{50}$, were used: 0.5 mM α-ketoglutarate, 1 mM PAH, 1 mM valproate, 5 mM urate and 5 mM folate. The initial uptake of [$^3$H]GlcA was measured at increasing GlcA concentrations in voltage clamped membranes with $K^+$/valinomycin in the absence and presence of unlabelled compounds. As illustrated in Fig. 3, all compounds showed a competitive mode of inhibition of glucuronic acid transport. The calculated $K_i$ for α-ketoglutarate, valproate and PAH were respectively 0.46 mM, 0.64 mM and 0.73 mM ($K_i = K_t[I]/\{(-1/x)-K_t\}$, in which $K_t$ is the $K_m$ for GlcA, [I] is the inhibitor concentration, $x$ is the intercept on the abscissa). For urate and folate, we found a $K_i$ of respectively 1.77 mM and 4.6 mM, indicating that these last compounds have a much lower affinity for the transporter.

## 4. Discussion

The lysosomal sialic acid transporter shows functional similarities with the MCT family [6]. Both, MCTs and our carrier, are energized by proton symport, have an overlap in substrate specificity in compounds like L-lactate, and are sensitive to specific inhibitors like cyano-cinnamates. The MCT family is classified as a subfamily of the major facilitator superfamily (MFS) [7,8]. This superfamily contains many different prokaryotic and eukaryotic anion transporters. Many homologues of bacterial genes have also been found in higher animals (mammalians). Members of the MFS are transporting small solutes, including sugars, often in response to ion gradients and are recently classified into 18 distinct subfamilies [8]. There can be significant overlap in substrate specificity between members of the different subfamilies.

In the experiments, described in this paper, several substrates of the OATs [9,10,16–18], like PAH, α-ketoglutarate, folate and valproate showed competitive *cis*-inhibition of GlcA uptake by the lysosomal sialic acid transporter. *Trans*-stimulation studies demonstrated that α-ketoglutarate is not only recognized but also actually translocated across the membrane by the mammalian sialic acid transporter. The OATs have homology with members of the sugar porter subfamily of the MFS [16,18]. Better phylogenetic analysis could reveal whether these transporters indeed belong to the sugar porter family. This shows that our observation of a mammalian carrier for both sugars and anions is not completely unexpected. We do not know whether compounds like α-ketoglutarate, PAH, urate, salicylate or valproate are physiological substrates in lysosomes or not. It would be interesting to study in more detail the role of the sialic acid transporter in the translocation of these drugs or their metabolites across the

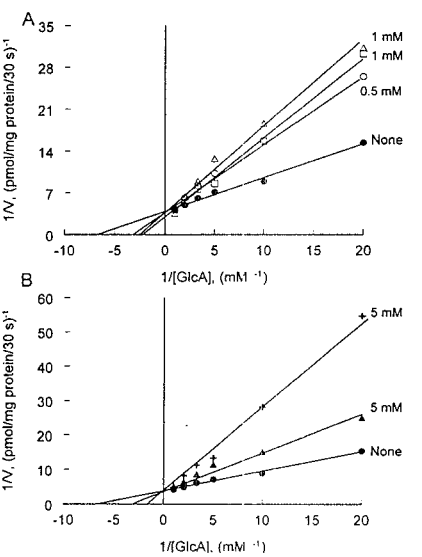


Fig. 3. Inhibition kinetics of [$^3$H]GlcA transport by different anions. Initial uptake rates of 10 μM [$^3$H]GlcA (30 s, 20°C) were measured at increasing GlcA concentrations in the presence of a proton gradient as described in the legend of Fig. 1. The uptake medium contained the following inhibitors: (A) 0.5 mM α-ketoglutarate (○), 1 mM PAH (□) or 1 mM valproate (Δ). (B) 5 mM of folate (▲) or urate (+). As a control, the uptake medium contained no inhibitor (●). The blank value was substracted from all determinations. Data are plotted double reciprocally.

lysosomal membrane in future experiments, in relation to Salla disease.

α-Ketoglutarate is also a known substrate of a different MFS family, the metabolite:$H^+$ symporter (MHS) family [8]. Substrates of these carriers all possess at least one carboxyl group and the carriers function as proton symporters.

Sialic acid is an important substrate of our transporter. Two putative sialic acid permeases are known in the prokaryotes *E. coli* [19] and *Haemophilus influenza*. They form the sialate:$H^+$ symporter (SHS) family [8]. Interestingly, the yeast *Saccharomyces cerevisiae* homologue (JEN1) of the *E. coli* permease [20], although functionally characterized as a carboxylic acid transport protein (lactate transport, [21]), was classified on the basis of sequence similarities as a member of this SHS family.

The hexuronate glucuronic acid is another main substrate of the lysosomal sialic acid transporter. Few other transporters for hexuronates and glucarate (saccharate) are known. They belong to the anion:cation symporter (ACS) family of the MFS [8]. Saccharic acid 1,4 and 3,6 lactons are strong *cis*-inhibitors of GlcA transport by the lysosomal sialic acid transporter [22]. The lysosomal sialic acid transporter is the only mammalian carrier known to transport free hexuronates like glucuronate, galacturonate and iduronate [1,6].

The aldonic acid sugars gluconate and galactonate are also recognized by our transport protein [1]. They are substrates of the recently identified gluconate:$H^+$ symporter family (GntP), which is not a member of the MFS [23].

The tested compounds methotrexate and folate are known substrates of another phylogenetic different superfamily, distinct from the MFS, called the organic anion transporting polypeptides (oatps) [24,25]. As shown in Fig. 1, methotrexate and folate mildly inhibited GlcA transport, with a very high $K_i$ for folate. Since the oatps do not recognize PAH and α-ketoglutarate and the lysosomal sialic acid transporter does not seem to transport or recognize with a high affinity substrates like methotrexate and folate, it is very unlikely that our transporter belongs to this family. Since sulfate, phosphate, glutamate, tetraethylammonium and several sugar-phosphates are not recognized by the lysosomal sialic acid transporter (*cis*-inhibition studies), the SulP family [11,12], the PHS family [11,13], the POT family [8,11], the OCT family [14] and the OPA family [15] are not of particular interest to us.

With the present studies we intended to extend the knowledge on the function of the lysosomal sialic acid carrier which can be helpfull in the identification of the transporter gene involved in SASD. Our recent biochemical studies suggested homology between this transporter and the monocarboxylate transporters, belonging to the MCT family. Here we present data which show functional similarities with α-ketoglutarate transporters within the OAT and MHS family. On its functional basis, the transporter could also belong to the SHS, ACS or GntP family. This means that the search for the human sialic acid transporter gene should not be limited to members of the MCT family.

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## PUBLICATION III

**Fibroblast silver loading for the diagnosis of Menkes disease.**

Verheijen, F.W., Beerens, C.E.M.T., Havelaar, A.C., Kleijer, W.J., and Mancini, G.M.S.

J. Med. Genet. 1998; 35:849-851

*J Med Genet* 1998;35:849–851

# Fibroblast silver loading for the diagnosis of Menkes disease

Frans W Verheijen, Cecile E M T Beerens, Adrie C Havelaar, Wim J Kleijer, Grazia M S Mancini

**Abstract**

**Menkes disease is a genetic disorder of copper metabolism. Copper uptake and retention assays on fibroblast or amniotic fluid cell cultures have been used for pre- and postnatal diagnosis. These copper loading tests are complicated by the use of $^{64}$Cu, which is not commonly available and has a very short (12.8 hours) physical half life. Besides copper, silver is also a substrate for the bacterial homologue of the Menkes transport protein. We report here that loading tests using radioactive silver ($^{110m}$Ag), instead of copper, can be used for the diagnosis of Menkes disease. $^{110m}$Ag is commercially available and has a convenient physical half life of 250 days, which makes it suitable for use in diagnostic laboratories. Our studies support the hypothesis that reduction of divalent to monovalent copper is an essential step preceding transport.**

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Keywords: Menkes disease; copper; silver

Menkes "steely hair" disease is a severe X linked recessive disorder of copper metabolism. Patients suffer from neurodegeneration, connective tissue abnormalities, and failure to thrive. Various copper dependent enzymes are deficient because of the malabsorption and decreased availability of copper in tissues. The disease generally leads to death in infancy or early childhood.[1 2]

The incorporation of $^{64}$Cu into cultured skin fibroblasts and amniotic fluid cells is used for pre- and postnatal diagnosis of Menkes disease.[3] After identification of the defective gene in 1993, diagnostic confirmation by mutation analysis of the Menkes gene (*ATP7A*, or MNK in earlier reports) is possible. However, the spectrum of mutations is broad, 80% of mutations being accounted for by small base pair changes.[4 5] Biochemical diagnosis remains essential, therefore, to confirm the clinical suspicion. The copper loading test requires particular expertise. $^{64}$Cu is not commonly available and its physical half life is very short (12.8 hours), which greatly complicates its use, and the test is carried out in only a few specialised centres world wide.

The *ATP7A* gene product is a putative copper transporter (ATP7A), highly homologous to copper transporters CopA and CopB from *Enterococcus hirae*.[1] A recent report that CopB also transports monovalent silver ions suggests that copper is transported by CopB as the reduced monovalent cation (CuI).[6] Also binding of copper as CuI has been shown for the in vitro expressed amino-terminal domains of ATP7A.[7] There is, as yet, no direct evidence of copper transport by ATP7A, but recent studies have shown an increased expression of ATP7A in Chinese hamster ovary cells resistant to excess copper.[8]

It has been shown that ATP7A is predominantly localised to the trans-Golgi apparatus, where it probably serves as an import protein for copper ions to provide copper to the cuproenzymes.[9–11] However, under conditions of copper excess a rapid shift in localisation from the Golgi to the plasma membrane is observed.[8] This ligand regulated trafficking of ATP7A can explain many clinical features of patients with Menkes disease, which are the result of the deficiency of several cuproenzymes (defective Golgi import of copper ions) or defective absorption by intestinal epithelial membranes. It was shown that, besides copper ions, silver ions were also able to induce this ligand regulated trafficking.[8] Since the bacterial homologue of ATP7A is able to transport copper and silver ions, we investigated whether ATP7A was also able to do so and consequently whether silver can replace copper in a more accessible biochemical test. $^{110m}$Ag is a commercially available β and γ emitter with a physical half life of 250 days and is therefore much easier to handle in a diagnostic laboratory.

We first investigated the incorporation of radioactive silver in cultured fibroblasts (fig 1). In controls and Menkes fibroblasts a clear, time dependent incorporation of $^{110m}$Ag was observed, with a higher incorporation in Menkes cells. Most of the radiolabel was released from the control cells during subsequent culturing in unlabelled medium for 24 hours ("chase"), while Menkes cells retained most radioactivity. The entire process was inhibited by copper sulphate added to the medium (data not shown). These results are comparable with previous data from $^{64}$Cu loading experiments.[3] Subsequently, cultured cells from 10 different Menkes disease patients were tested with this silver incorporation test. As shown in table 1, cell lines from Menkes disease patients showed a higher average level of silver incorporation during the first 16 hours as compared with controls. During the subsequent chase period, the Menkes cell lines retained high levels of radioactivity, ranging from 77 to 100% of the level before chase, compared to 14 to 48% in 10 controls. This shows that silver incorporation perfectly discriminates Menkes cells from

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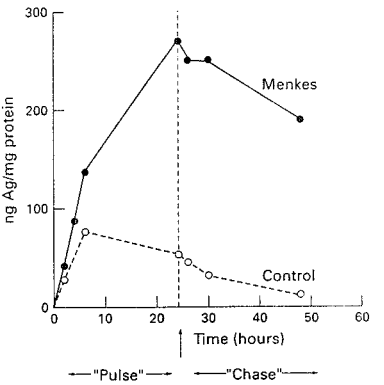


*Figure 1 Time dependent incorporation of $^{110m}$Ag in cultured fibroblasts from a control and from a Menkes disease patient. Cells were harvested at the indicated time points ("pulse" period). After 24 hours' loading, the radioactive medium was replaced by fresh, non-silver containing medium and cells were further cultured for the indicated time ("chase" period). For details of the assay see description below table 1.*

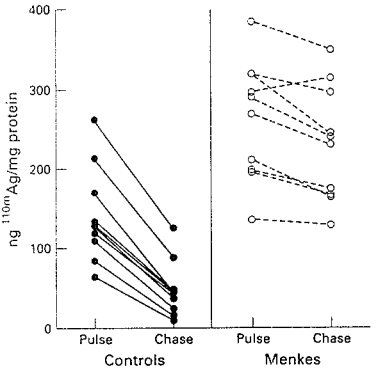


*Figure 2 Silver incorporation by 10 different control and 10 different Menkes fibroblast cultures. The incorporation values, obtained after 16 hours' "pulse" and after subsequent 24 hours' "chase" are reported for each cell strain.*

controls. Cultured amniotic fluid cells from a fetus with Menkes disease show entrapment of $^{110m}$Ag after 24 hours' chase (table 1), suggesting that the silver loading test can be used for prenatal diagnosis as well.

In fig 2, the individual incorporation levels after pulse and chase are plotted for all cell strains. After the first 16 hours there is some overlap of the $^{110m}$Ag levels in Menkes and control cells. However, during the subsequent 24 hours' chase all control cells rapidly release $^{110m}$Ag, while all Menkes cells show no or very little efflux. Evidently the 16 hours loading levels are the result of simultaneous import and efflux processes, which are in part dependent on the rate of uptake. In contrast, chase experiments directly address the efflux. Since in Menkes disease fibroblasts the efflux of copper (and silver) is defective and not the uptake, diagnostic conclusions can be drawn best from the chase experiments.

The long half life of $^{110m}$Ag and its common availability will potentially facilitate both diagnosis and research aiming at improved therapy of Menkes disease and its allelic variant occipital horn syndrome.[2] The finding that CopB (*Enterococcus hirae*) and ATP7A (human), both members of a family of copper transporting P type ATPases, also transport monovalent silver[6] (this paper) suggests that other members of this family might also transport silver. Among these are the transporters involved in Wilson disease and Indian childhood cirrhosis.[12] It is not known whether the transport of silver is merely a biochemical property of the substrate binding site of copper transporters or that silver transport has a physiological role. There are only sporadic reports suggesting a specific role for silver ions in the oxidative burst of polymorphonuclear leucocytes.[13]

A few Menkes disease patients respond to copper supplementation whereas others do not. It is hypothesised that in some patients with residual copper transport, early copper supplementation therapy can be beneficial.[14] In one of the mouse models for Menkes disease, the brindled mouse, the activity of copper dependent lysyl oxidase dramatically increases after subcutaneous injection of reduced copper (CuI). This suggests that the effectiveness of copper supplementation depends on its oxidation state.[15] Copper loading tests in nine different mottled mouse variants do not correlate genotype with phenotype.[16] Our data support the hypothesis that copper is transported as a monovalent cation by ATP7A and that copper reduction represents an essential step preceding transport. Thus, comparison of copper and silver transport kinetics in different mutants might help to elucidate whether, in some of these mutants responsive to CuI supplementation therapy, the transport defect is secondary to a defective reduction mechanism.

*Table 1 Diagnosis of Menkes disease by silver loading of cell cultures*

| | *Pulse (16 h)* | *Chase (24 h)* | *% Retained* |
|---|---|---|---|
| Fibroblasts | | | |
| Controls (n=10) | | | |
| Mean (SEM) | 141.2 (18.8) | 47.5 (11.0) | 30 ± 3%<br>Range 14–48% |
| Menkes (n=10) | | | |
| Mean (SEM) | 261.7 (23.8) | 230.7 (23.0) | 88 ± 2%<br>Range 77–100% |
| Amniocytes | | | |
| Control 1 | 14.2 | 3.7 | 26% |
| Control 2 | 33.0 | 3.3 | 10% |
| Menkes | 33.2 | 30.7 | 93% |

Each cell line was seeded to confluency in four separate wells of six well plates (Costar, 9.6 cm²/well). The cells were cultured for three to four days in F10* medium (+ 15% FCS + PS) in a 3% $CO_2$ incubator at 37°C. To start the experiment, the medium was replaced by 2 ml fresh medium containing 1.25 µmol/l $^{110m}AgNO_3$ (Amersham spec act 2.7 µCi/µg Ag). After 16 hours wells were rinsed twice with 4 ml PBS. Two wells were harvested by lysis with 500 µl 0.2% SDS in 0.2 mol/l NaOH. The other two wells were subsequently cultured for 24 hours in fresh medium without label before harvesting. The lysates were neutralised and samples were taken for protein determination and liquid scintillation counting.
All Menkes cell lines exhibited increased accumulation in previous $^{64}$Cu loading tests (The John F Kennedy Institute, Copenhagen). Loading is expressed as ng $^{110m}$Ag/mg protein (average of duplicate wells). % Retained = chase/pulse * 100%.

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## PUBLICATION IV

**Characterization of a heavy metal ion transporter in the lysosomal membrane.**

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# Characterization of a heavy metal ion transporter in the lysosomal membrane

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**Abstract** **Lysosomes are thought to play a role in various aspects of heavy metal metabolism. In the present study we demonstrate for the first time the presence of a heavy metal ion transport protein in the lysosomal membrane. Uptake of radioactive silver both in highly purified lysosomal membrane vesicles and in purified intact lysosomes showed the typical kinetics of a carrier-mediated process. This transport was stimulated by ATP hydrolysis, and showed specificity for $Ag^+$, $Cu^{2+}$, and $Cd^{2+}$. All biochemical properties of this lysosomal metal ion transporter could classify it as a heavy metal transporting P-type ATPase. Long Evans Cinnamon (LEC) rats, an animal model for the copper transport disorder Wilson disease, showed normal lysosomal silver transport.**



*Key words:* Lysosomal transporter; Heavy metal ion; Copper; Silver; P-type ATPase; Wilson disease

## 1. Introduction

Lysosomes are intracellular acid organelles which are mainly responsible for the degradation of a variety of biological macromolecules, derived from both extra- and intracellular constituents. Various specific transport systems have been characterized in the lysosomal membrane either for the release of small degradation products or for the uptake of small substrates [1]. Previously, we have developed a method for studying transport across the lysosomal membrane using highly purified lysosomal membrane vesicles. With this method, we have characterized a sialic acid transporter, a glucose transporter, and a chloride channel in the lysosomal membrane [2–4]. For many years, lysosomes are also thought to play a role in various aspects of the metabolism of heavy metals. For instance, during hepatic copper overload the major route of copper excretion is via exocytosis of lysosomal contents into biliary canaliculi [5]. However, so far, direct evidence that lysosomes are able to take up or exclude, sequester and mobilize heavy metal ions by specific transporters has been lacking. The importance of mechanisms regulating copper metabolism is shown by the occurrence of severe diseases like Menkes and Wilson disease. Both diseases are caused by genetic defects in distinct steps of copper metabolism [6]. Transport studies using radioactive copper are limited by its availability and short physical half-life (12.8 h). Recently, we have shown that radioactive silver can replace copper in copper transport studies [7]. This provides an excellent opportunity to study copper transport mechanisms. In this paper we demonstrate the presence of a heavy metal ion transport protein in the lysosomal membrane. This transport protein is the first heavy metal ion transporter detected in the lysosomal membrane.

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*Abbreviations:* VacA, vacuolating cytotoxin A; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; DMSO, dimethyl sulfoxide; NaBu, sodium butyrate; EGF, human epidermal growth factor

## 2. Materials and methods

### 2.1. Materials

$^{110m}Ag$ was purchased from Amersham (specific activity of 1 μCi/μg Ag). Seven-week-old Long Evans Cinnamon (LEC) rats were purchased from Charles River Japan. All chemicals used were obtained from Sigma or as indicated.

### 2.2. Preparation of lysosomal membranes vesicles and intact lysosomes from rat liver

Highly purified lysosomal membrane vesicles were isolated from livers of adult Wistar rats or 7-week-old LEC rats, as described earlier [2]. Characteristics of LEC rats are described elsewhere [8]. The lysosomal membrane vesicles were suspended at a protein concentration of 8–10 mg/ml in 50 mM KHEPES, pH 7.4, and were stored at −70°C. Intact lysosomes were isolated from a liver of an adult Wistar rat by Percoll gradient centrifugation [9]. The lysosomal/mitochondrial pellet of the above described procedure for lysosomal membrane vesicles was resuspended in 5 ml 0.25 M sucrose/50 mM KHEPES, pH 7.4, and was slowly loaded onto a Percoll gradient. The gradient was made of 40% Percoll (Pharmacia) in 0.25 M sucrose/50 mM KHEPES, pH 7.4. After 1 h centrifugation in a Beckmann Ti 45 rotor (fixed) at 20000×*g* at 4°C, the gradient was divided in fractions of 1 ml. In all fractions the activity of the lysosomal enzyme β-hexosaminidase was determined, as described [10]. Fractions enriched in β-hexosaminidase (60–80-fold over total homogenate) were combined (±10 ml), diluted 6.5 times with 0.25 M sucrose/50 mM KHEPES, pH 7.4, to dilute the Percoll concentration and centrifuged for 20 min at 8000×*g* in Ti 45 rotor at 4°C. The pellet was used for transport assays, performed immediately after preparation. Latency of the lysosomes was based on measurements of the activity of the intralysosomal enzyme β-hexosaminidase in the presence and absence of the detergent Triton X-100. The lysosomal latency of β-hexosaminidase was approximately 72%.

### 2.3. Transport assays

Transport of $^{110m}Ag$ was measured into lysosomal membrane vesicles or intact lysosomes. For the transport assays using lysosomal membrane vesicles, the frozen membrane vesicles (70–120 μg of protein in 10 μl) were quickly thawed and pre-incubated with 50 mM KHEPES, pH 7.4/10 mM reduced glutathione/5 μM valinomycin (Boehringer Mannheim)/5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) for 10 min at room temperature (total volume 25 μl). Glutathione (GSH) was added to reduce aspecific binding of Ag to the membrane. The ionophores valinomycin and FCCP were added to prevent, respectively, the formation of a membrane potential and the formation of a proton gradient due to stimulation of the lysosomal $H^+$-ATPase by ATP. Simultaneously, radiolabeled $^{110m}Ag$ (0.032 μCi) was pre-incubated with 10 mM reduced glutathione for 10 min at room temperature to allow the formation of an Ag-GSH complex (1:1, total volume 10 μl). After 10 min pre-incubation, the


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suspensions were pre-warmed at 37°C for 3 min. The uptake experiments at 37°C were started by adding a 5-μl aliquot of 32 mM MgATP in 50 mM KHEPES, pH 7.4, to a 10-μl aliquot of the $^{110m}$Ag/GSH suspension and subsequently to a 25-μl aliquot of the pre-incubated membrane suspension. In control experiments ATP was replaced by AMP (Boehringer Mannheim). Transport was terminated by the addition of 60 μl of ice-cold stop-solution (50 mM KHEPES, pH 7.4) and 100 μl were immediately applied to a Sephadex G50 fine (Pharmacia LKB) column (Pasteur pipettes, 0.5×5 cm), equilibrated in cold stop-solution at 4°C. Vesicles were eluted with 1 ml ice-cold stop-solution. Vesicle-associated radioactivity was determined by liquid scintillation counting in 10 ml Instagel (Packard). Aspecific binding of $^{110m}$Ag to the membrane was determined either by 0 min incubations at 0°C (Fig. 1A) or by incubations at 37°C in the presence of high concentrations $CuSO_4$ (>100 μM, as indicated in the legends), and subtracted from all determinations. Transport assays using intact lysosomes were largely performed as described above for the lysosomal membrane vesicles. However, 20-μl aliquots of intact lysosomes were used and all buffers contained 0.25 M sucrose. Inhibitors, like $CuSO_4$, $AgNO_3$, or other metal sulfates were applied to the ATP solution and were added just before the start of the assay. In the competitive inhibition experiments the 25 μM unlabeled $CuSO_4$ and $CdSO_4$ were added to the $^{110m}$Ag/GSH suspension. The ATPase inhibitors and protein modifier 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) were added to the pre-incubation solution of the lysosomal membrane vesicles. All experiments were performed in duplicate.

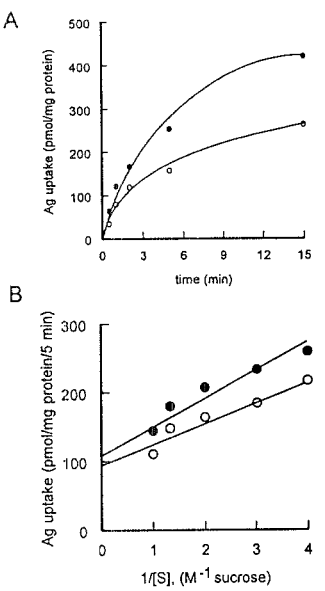


Fig. 1. A: $^{110m}$Ag uptake in lysosomal membrane vesicles is stimulated by ATP. Membrane vesicles (100 μg of protein) were pre-incubated at 20°C for 10 min in medium containing 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 μM valinomycin, and 5 μM FCCP. Assays at 37°C were started by the addition of 7.5 μM $^{110m}$Ag, 10 mM GSH in the presence (●) or absence (○) of 4 mM ATP. All data are corrected for aspecific binding as measured at 0 min incubation. B: Effect of increasing medium osmolarity by sucrose on Ag uptake in lysosomal membrane vesicles. Lysosomal membrane vesicles were pre-incubated in 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 μM valinomycin, 5 μM FCCP, and with 0.25–1 M sucrose for 30 min at 20°C. Vesicles were then incubated for 5 min at 37°C with 7.5 μM $^{110m}$Ag in 50 mM KHEPES, pH 7.4, 10 mM GSH, 0.25–1 M sucrose and in the presence (●) or absence (○) of 4 mM ATP.

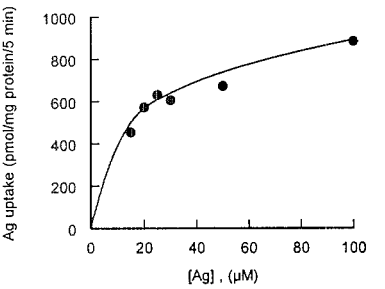


Fig. 2. Carrier mediated uptake of $^{110m}$Ag into lysosomal membrane vesicles. Lysosomal membrane vesicles were pre-incubated as described in Fig. 1A. $^{110m}$Ag uptake (5 min, 37°C) was measured in the presence of 4 mM ATP and increasing concentrations of Ag, and corrected for aspecific binding as measured in the presence of 100 μM $CuSO_4$.

## 3. Results

### 3.1. Carrier mediated uptake of $^{110m}$Ag in lysosomal membrane vesicles

Studies on copper (Cu) transport have been greatly complicated by the $^{64}$Cu isotope, which is not readily available and has a very short physical half-life (12.8 h). Recently, we have shown that copper transport can be easily measured using radioactive silver (Ag) [7]. $^{110m}$Ag is commercially available and has a physical half-life of 250 days. We used this isotope to investigate the presence of a heavy metal ion transporter in the lysosomal membrane. Since, so far, all characterized copper transporters are belonging to the class of P-type ATPases [11], we investigated the transport of $^{110m}$Ag in the presence and absence of ATP. It is known that heavy metal ions can easily bind to proteins, disturbing measurements of membrane transport of these ions [12]. This aspecific binding can be reduced by the addition of glutathione (GSH) [13]. Therefore, in all our transport assays GSH was present. Appreciable uptake of $^{110m}$Ag was observed in rat liver lysosomal membrane vesicles. Fig. 1A shows that Ag uptake (7.5 μM) was stimulated by ATP. To determine whether the amount of Ag observed in uptake assays is due to real uptake (internalization) or binding on the outside membrane, osmotic shrinking experiments were performed. In these experiments increasing of the medium osmolarity leads to shrinking of the vesicles (i.e. the internal volume gets smaller, while the membrane surface is constant). As shown in Fig. 1B, the amount of Ag associated with the vesicles decreased with increasing osmolarity of the external medium. This indicated that Ag is transported into an osmotically active intravesicular space. Extrapolation of these data to an infinite high medium osmolarity (i.e. a negligible intravesicular volume) revealed the amount of $^{110m}$Ag which is not taken up, but which is present bound to the outside membrane. The same binding component is seen in control assays (both lines of Fig. 1B cross the Y-axis at approximately the same point). Furthermore, the same aspecific binding component was observed when assays

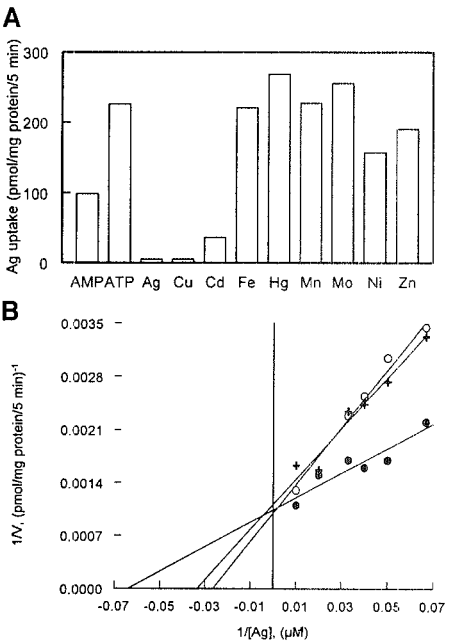


Fig. 3. A: *Cis*-inhibition of metal ions on the ATP stimulated uptake of $^{110m}$Ag. Lysosomal membrane vesicles were incubated for 5 min at 37°C in the presence of 7.5 μM $^{110m}$Ag, 50 mM K.HEPES, pH 7.4, 10 mM GSH, 5 μM valinomycin, 5 μM FCCP, 4 mM AMP or ATP and 100 μM of the indicated metal sulfates. Values are mean ± S.D. of two experiments performed in duplicate and corrected for aspecific binding as measured in the presence of 100 μM $CuSO_4$. B: Competitive inhibition of $^{110m}$Ag transport by $CuSO_4$ and $CdSO_4$. Initial uptake rates of 15 μM $^{110m}$Ag were measured at increasing Ag concentrations in pre-incubated lysosomal membrane vesicles, as described in Fig. 1. Data were corrected for aspecific binding as measured in the presence of 500 μM $CuSO_4$. Data were plotted double reciprocally, without inhibitor (●), with 25 μM unlabeled $CuSO_4$ (○) or with 25 μM unlabeled $CdSO_4$ (+). As reported in the text $K_i$s were calculated by the following equation: $K_i = K_t[I]/\{(-1/x)-K_t\}$ ($K_t$ is the $K_m$ for Ag, [I] is the inhibitor concentration, $x$ is the intercept on the abscissa).

were performed in the presence of high concentrations ( > 100 μM) unlabeled $AgNO_3$ or $CuSO_4$ (data not shown). Therefore, in all subsequent experiments assay blanks were determined in the presence of high concentrations of $CuSO_4$. Next, we determined if transport rates of $^{110m}$Ag uptake were saturable. Initial uptake of $^{110m}$Ag was studied under zero-*trans* conditions at increasing Ag concentrations in the presence of ATP. All data were corrected for an aspecific binding component as measured in the presence of high concentrations of inhibitor. We observed the typical kinetics of carrier mediated transport by one single process, with an apparent affinity constant $K_t$ of 16 μM in the presence of ATP (Fig. 2).

### 3.2. Substrate specificity of the lysosomal heavy metal ion transporter

To determine the substrate specificity of the transporter, we first tested the *cis*-inhibition effects of several metal ions. A clear *cis*-inhibition of $^{110m}$Ag uptake was seen with $Ag^+$, $Cu^{2+}$ and $Cd^{2+}$, but not with $Fe^{2+}$, $Hg^{2+}$, $Mn^{2+}$, $Mo^{4+}$, $Ni^{2+}$, and $Zn^{2+}$ (Fig. 3A). To determine the mode of inhibition, initial uptake of $^{110m}$Ag was measured at increasing Ag concentrations in voltage clamped membranes with $K^+$/valinomycin, in the absence and presence of, respectively, unlabeled $CuSO_4$ or $CdSO_4$. The results were fitted to a double reciprocal plot, showing a clear mode of competitive inhibition of $CuSO_4$ ($K_i$ of 17 μM) and of $CdSO_4$ ($K_i$ of 28 μM) on $^{110m}$Ag transport (Fig. 3B). These results demonstrated that Ag, Cu, and Cd are recognized by the same protein in the lysosomal membrane.

### 3.3. Ag uptake by lysosomal membrane vesicles is stimulated by ATP hydrolysis

So far, all known copper transporters are P-type ATPases [11]. We investigated if the lysosomal heavy metal ion transporter also belongs to this group of P-type ATPases. To test if ATP stimulation of Ag uptake by lysosomal membrane vesicles is dependent on ATP hydrolysis, we tested two non-hydrolyzable analogues of ATP, adenosine 5′-[β,γ-methylene]-triphosphate tetralithium (AMP-PCP) and 5′-adenylylimidodiphosphate (AMP-PNP). Both ATP analogues did not stimulate Ag uptake in comparison to the control level (AMP level) (Table 1). This indicated that ATP hydrolysis is required for the stimulation of Ag uptake by lysosomal membrane vesicles. Besides ATP, also other triphosphates, CTP and GTP, but not the monophosphates AMP, CMP, and GMP were able to stimulate Ag uptake, indicating that generally hydrolysis of high energy phosphate bonds is required for transport (Table 1). The effect of specific ATPase inhibitors and protein modifiers on the Ag uptake by the lysosomal membrane vesicles was investigated. Vanadate, acting as a phosphate analogue, is considered to be a specific inhibitor of several P-type ATPases [14], inhibiting ATP hydrolysis at micromolar concentrations. As shown in Table 2, Ag transport by the lysosomal carrier was not inhibited by vanadate. This is in accordance with an earlier report [15] that P-type

Table 1
Effects of monophospho- and triphosphonucleotides, and non-hydrolyzable ATP analogues on Ag uptake into lysosomal membrane vesicles

| Tested compound | Transport activity | |
|---|---|---|
| | pmol/mg/5 min | % of control |
| None[a] | 92.5 ± 0.7 | |
| Nucleotide | | |
| ATP | 145.8 ± 18.6 | 158 |
| CTP | 148.2 ± 0.6 | 160 |
| GTP | 115.4 ± 24.8 | 125 |
| AMP | 83.7 ± 6.8 | 91 |
| CMP | 89.4 ± 4.9 | 97 |
| GMP | 78.1 ± 21.4 | 85 |
| Non-hydrolyzable ATP-analogues | | |
| AMP-PCP | 89.6 ± 12.0 | 97 |
| AMP-PNP | 77.6 ± 14.4 | 84 |

[a]Net uptake in the presence of 50 mM K.HEPES, pH 7.4, corrected for aspecific binding as measured in the presence of 100 μM $CuSO_4$, was set to 100%. All uptakes are performed in the presence of 4 mM of the indicated compounds, incubated for 5 min at 37°C and are corrected for aspecific binding.

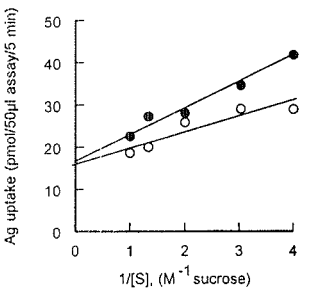


Fig. 4. Effect of increasing medium osmolarity by sucrose on Ag uptake in intact lysosomes. Percoll gradient isolated intact lysosomes were pre-incubated in 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 µM valinomycin, 5 µM FCCP, and with 0.25–1 M sucrose for 1 h at 20°C. Lysosomes were then incubated for 5 min at 37°C with 15 µM $^{110m}$Ag in 50 mM KHEPES, pH 7.4, 10 mM GSH, 0.25–1 M sucrose and in the presence (●) or absence (○) of 4 mM ATP.

ATPases with a specificity for heavy metal ions seem to be resistant to vanadate inhibition. None of the other tested ATPase inhibitors inhibited Ag uptake (Table 2). Surprisingly, only the protein modifier DIDS showed a slight inhibition of transport in the presence of ATP. In fact, inhibition of transport was observed to the level of control transport (AMP). Apparently, ATP stimulation was inhibited by DIDS. This indicated that lysine residues (modified by DIDS) may play a role in the binding of ATP (anion).

### 3.4. $^{110m}$Ag uptake in purified intact lysosomes

All our previous studies made use of lysosomal membrane vesicles. Such vesicles are a mixed population of inside-out and right-side-out vesicles [2]. To investigate if the lysosomal heavy metal ion transporter functions physiologically as an importer or exporter, we performed uptake studies using intact lysosomes. These highly purified lysosomes, isolated by Percoll gradient centrifugation, showed ATP stimulated $^{110m}$Ag uptake similar to that observed in lysosomal membrane vesicles. The increase of the external medium osmolarity by the addition of sucrose (leading to shrunken lysosomes) resulted in a concomitant decrease of $^{110m}$Ag uptake (Fig. 4), demonstrating import into the intralysosomal compartment.

### 3.5. The lysosomal heavy metal ion transporter is not affected in an animal model for Wilson disease

The Long Evans Cinnamon (LEC) rat is a biochemical and genetic animal model for human Wilson disease [16,17]. The gene mutated in this disease normally encodes for a copper P-type ATPase. The LEC rats show a reduction in the rate of incorporation of copper into ceruloplasmin and a reduction in the biliary excretion of copper. A decreased biliary copper secretion due to a lysosomal defect has been suggested for Wilson disease [5,18,19]. Therefore, we investigated the Ag transport in lysosomal membrane vesicles of 7-week-old LEC rats. No significant difference in $^{110m}$Ag transport was observed in vesicles from both normal and LEC rats (160 and 228 nmol/mg/1 min incubation and 381 and 349 nmol/mg protein/5 min incubation, respectively). Apparently, this newly described lysosomal heavy metal ion (copper) transporter is not affected.

## 4. Discussion

In the present study we provide biochemical evidence for the presence of a heavy metal ion transport system in the lysosomal membrane stimulated by ATP hydrolysis. Our studies on Ag transport were complicated by aspecific binding of the free metal ions to proteins. Addition of excess glutathione was necessary to reduce the aspecific binding of Ag to the membranes. Similar problems have been encountered with copper transport across biological membranes [13,20,21]. In our experiments, at least 60% of the vesicle-associated silver was the result of carrier-mediated transport into an osmotically sensitive vesicle.

The heavy metal ions, Ag, Cu, and Cd competitively inhibited $^{110m}$Ag uptake into the lysosomal membrane vesicles, while many others did not. This demonstrates that the lysosome contains a carrier with a specificity for a limited number of metal ions. This carrier is different from the recently identified general metal-ion carrier, DCT1 (divalent-cation transporter), which has a much broader substrate specificity, including Fe, Zn, Mn, Co, Cd, Cu, Ni and Pb, and is present in the plasma membrane [22]. While Cd is only occurring as a divalent cation and Cu as a mono- or divalent cation, Ag is only occurring as a monovalent cation. Hence, this lysosomal transport system apparently does not discriminate monovalent from divalent ions. We assume that Ag forms a complex with GSH and that this complex is recognized by the transport protein. Since the lysosomal membrane is impermeable to

Table 2
Effect of ATPase inhibitors and protein modifiers on Ag uptake into lysosomal membrane vesicles

| Tested compound | Concentration (mM) | Transport activity | | Target |
|---|---|---|---|---|
| | | pmol/mg/5 min | % of uptake | |
| ATP | 4 | 307.1 ± 4.2 | – | – |
| AMP | 4 | 178.5 ± 20.1 | 58 | – |
| ATP+$KNO_3$ | 50 | 313.8 ± 28.5 | 102 | V-type ATPase |
| ATP+*N*-ethylmaleimide | 1 | 335.5 ± 53.9 | 109 | V-type ATPase |
| ATP+bafilomycin A1 | 0.001 | 283.9 ± 9.3 | 93 | V-type ATPase |
| ATP+$NaN_3$ | 5 | 333.6 ± 30.4 | 109 | F-type ATPase |
| ATP+$VO_4^{3-}$ | 0.1 | 296.7 ± 30.2 | 97 | P-type ATPase |
| ATP+DIDS | 1 | 156.3 ± 27.9 | 51 | Anion carriers |

Assays were performed in the presence of ATP or AMP or in the presence of ATP and the indicated compounds for 5 min at 37°C and corrected for aspecific binding as measured in the presence of 100 µM $CuSO_4$. The uptake in the presence of ATP was set to 100%.

GSH [23], Ag is released from the complex, and is transported as a free monovalent ion into the vesicles.

Based on sequence similarities, about 20 putative copper ATPases have been identified from various sources [11], all belonging to the subclass of heavy metal ion P-type ATPases, i.e. the CPx-type ATPases (based on a conserved intramembranous cysteine-proline-cysteine or cysteine-proline-histidine motif). Direct evidence for a function in copper transport exists only for the CopB ATPase (*Enterococcus hirae*) [24] and for ATP7A (human), which is defective in Menkes disease [7]. Both proteins can also transport silver.

The lysosomal heavy metal ion transporter is stimulated by ATP, but not by non-hydrolyzable ATP analogues. This indicates that hydrolysis is needed for stimulation. This, together with its insensitivity to vanadate suggests that the lysosomal transporter belongs to the CPx-type ATPases. Ag uptake was not only stimulated by ATP, but also by other triphosphonucleotides (i.e. CTP and GTP). To our knowledge, stimulation by CTP or GTP has not been tested earlier for the CPx-type ATPases. However, it is known that other ATP-dependent transport systems can be stimulated by different triphosphonucleotides [25].

In our studies the metals (Ag, Cu, and Cd) are supplied to the lysosomal transporter as complexes with GSH. Several transport systems have been demonstrated for the transport of GSH-complexes (e.g. the canalicular multispecific organic anion transporter (cMOAT)) [26]. However, a similar GSH-complex transport is unlikely in our studies for the following reasons: (i) our lysosomal system recognizes also monovalent ions ($Ag^+$), while substrates of cMOAT are supposed to have at least two negative charges (e.g. $GS^-..^+Zn^+..^-SG$) [27]; (ii) the lysosomal transporter has a much more restricted substrate specificity for Ag, Cu, and Cd, with competitive inhibition among different metal ions; (iii) moreover, the lysosomal membrane is reported to be impermeable to GSH [23].

It is interesting to speculate on a possible physiological function of this new lysosomal heavy metal ion transporter. Both ATP stimulation (the intra-lysosomal lumen does not contain ATP) and transport into intact lysosomes suggest that this carrier functions as a lysosomal importer for copper and cadmium, with an extralysosomal ATP-binding site. Silver, which is also recognized by this importer, is not known to have any physiological function. Under certain conditions, import of copper may be required for storage or disposal. A decreased biliary excretion of copper leads to hepatic accumulation of this heavy metal. Several hypotheses, including defective transporters, have been proposed to explain the defective biliary copper excretion in Wilson disease [28]. It has been demonstrated that in conditions of hepatic overload, the major route for biliary copper excretion is exocytosis of lysosomal contents into biliary canaliculi [5]. Since the transport observed in LEC rats, an animal model for Wilson disease, was not affected, this new lysosomal heavy metal ion transporter does not play a role in the release of copper into the bile. However, it may explain the mechanism by which copper is taken up into the lysosomes during overload conditions [5].

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## Chapter 4 RECENT DEVELOPMENTS AND FUTURE APPROACHES

**4.1 Characterization of the first lysosomal membrane transporter for heavy metal ions**

**4.2 A functional approach towards the gene for sialic acid storage disease**

## 4.1 Characterization of the first lysosomal membrane transporter for heavy metal ions

For many years, lysosomes have been known to play a role in the regulation of the intracellular homeostasis of heavy metal ions. The biochemical characterization of a heavy metal ion transporter in the lysosomal membrane (publication IV) provided the first direct experimental evidence that lysosomes are able to take up and possibly sequester heavy metal ions. This lysosomal transporter has the kinetic characteristics of a heavy metal P-type ATPase and is responsible for the uptake of silver, copper, and cadmium ions. The heavy metal ion P-type ATPase family comprises members with a specificity for either silver and copper or cadmium ions (Solioz and Vulpe, 1996). Several other transporter families have been identified which include heavy metal ion transporters (Saier, 1998). These families are present in prokaryotes as well as eukaryotes and at different cellular localizations. Therefore, the lysosomal membrane may contain other heavy metal ion transporters with a different specificity. There are suggestions for an iron export mechanism, since ferritin is known to be degraded in lysosomes (Ringeling et al., 1989; Radisky and Kaplan, 1998).

Lysosomes also play a role in diseases in which heavy metal homeostasis is disturbed. In LEC rats, the animal model for Wilson disease, we excluded that the lysosomal heavy metal ion transporter is defective, although we cannot exclude that the mobilization or excretion of the lysosomal copper into the bile is defective in this disease. Recently, it has been demonstrated that the biliary excretion of copper via lysosomes is restored in LEC rats after the introduction of the *ATP7B* gene in these rats, which indicates the participation of ATP7B in the lysosomal-biliary excretory pathway for copper (Terada et al., 1999). Accumulation of copper in lysosomes is also observed in other copper overload diseases, including the human disorders endemic Tyrolean infantile cirrhosis and Indian childhood cirrhosis, and the copper toxicosis in Bedlington terriers (Alt et al., 1990; Adamson et al., 1992; Haywood et al, 1996). It would be interesting to test lysosomal heavy metal transport with radioactive silver in these disorders. Technical difficulties in obtaining enough material (lysosomal membranes from patient livers) limit at the moment the realization of this test in the human disorders, but perhaps the canine model can be helpful.

## 4.2 A functional approach towards the gene for sialic acid storage disease

The purification of the lysosomal sialic acid transporter initiated a number of detailed studies aimed at understanding the physiological function of this transporter. The transport activity correlated with a 57 kDa protein isolated from rat liver lysosomes. Characterization of the purified protein revealed that besides acidic monosaccharides also other (non-sugar) mono- and dicarboxylated anions are transported by this protein. In

other words, the lysosomal sialic acid transporter has a wide substrate specificity for carboxylated anions. In this respect the sialic acid transporter is quite unique among the lysosomal transport proteins, since most of these appear to have a very restricted substrate specificity (see chapter 1.2). However, comparison at the molecular level is not possible since none of the other transporters has been isolated. It would be interesting to know whether the wide substrate specificity of the sialic acid transporter has implications for the pathophysiology. So far, a defective transport of sialic acid and glucuronic acid has been established in lysosomal membrane vesicles of fibroblasts from patients with sialic acid storage disease (SASD) (Mancini et al., 1991). Of these acidic sugars, sialic acid has been demonstrated to accumulate extensively in patient's lysosomes, whereas only a slight lysosomal accumulation of glucuronic acid has been observed (Gahl et al., 1995). Studies on the contribution of different anions in the pathophysiology of SASD are lacking and might provide new insight into the cause of clinical heterogeneity. A reduction in the turnover rate of gangliosides, sialioglycoconjugates and sphingolipids has been observed in SASD fibroblasts (Chigorno et al., 1996; Pitto et al., 1996). It is possible that the disturbance of myelination in severe Salla disease patients, observed with brain imaging (MRI) and pathological studies, is due to lysosomal accumulation of gangliosides, the main sialic acid-containing sphingolipids and the major component of myelin (Autio-Harmainen et al., 1988; Haataja et al., 1994b).

On the basis of the extensive functional characterization, we conclude that the sialic acid transporter belongs to one of the selected anion transporter families with overlapping substrate specificities (publication II). Those families which comprise anion as well as sugar transporters are most interesting. This detailed functional characterization of the lysosomal sialic acid transporter provides a clear-cut approach towards the identification of the gene involved in SASD, by searching in EST (expressed sequence tag) databases for members of these anion transporter families. Transporter genes from these families, which map in the known critical SASD region on 6q14-q15 (Leppänen et al., 1996) should be considered as strong functional candidates and should be tested for mutations in SASD patients. By this "functional candidate gene approach" several ESTs that might encode novel anion transporters were mapped by hybrid panel hybridization and fluorescent in situ hybridisation. They were excluded as candidate genes for SASD, since they did not map in the critical SASD region (unpublished results).

Many new EST clones have been mapped on the recent human genome map (GeneMap 98)(Deloukas et al., 1998). Some of these EST clones mapped near markers in the critical region of SASD. Among these, some overlapping ESTs showed significant homology to mammalian $Na^+$-phosphate and bacterial $H^+$-hexuronate cotransporters, both members of the anion:cation symporter (ACS) family. These ESTs provided the opportunity to clone the cDNA of this functional candidate (Verheijen et al., manuscript in preparation). The predicted size of the protein encoded by this cDNA (495 amino acids), is in agreement with the molecular mass of our purified sialic acid transport

protein, observed on SDS-PAGE gel as 57 kDa. The protein contains 7 potential N-glycosylation sites and 12 transmembrane domains, characteristic for a transporter of the major facilitator superfamily (MFS) to which the ACS family belongs. The ACS family contains not only anionic sugar transporters, but also $Na^+$-phosphate symporters (Pao et al., 1998). Mutation analysis of this cDNA identified a possible founder mutation in the Finnish patients with Salla disease and a number of deletions and insertions in the infantile phenotype (ISSD) (Verheijen et al., manuscript in preparation). The novel gene has a high homology to a brain-specific $Na^+$:phosphate symporter (Ni et al., 1996). The gene of this brain phosphate transporter is localized at chromosome 19q13.3, a susceptibility locus for late onset Alzheimer disease (Ni et al., 1996). Several reports have demonstrated that phosphate homeostasis is important in the central nervous system (Ni et al., 1994; Argov et al., 1997; Bellocchio et al., 1998). Patients with Salla disease always show severe mental retardation and cognitive regression, even in the absence of further neurologic abnormalities.

Future expression studies are essential to investigate whether the SASD gene product can function as an acidic monosaccharide:$H^+$ symporter and perhaps also as a $Na^+$:phosphate symporter, particularly in neuronal cells. As a $Na^+$:phosphate symporter it should reside in membranes different from the lysosomal membrane, since lysosomes *in vivo* do not contain a sodium gradient across their membranes. Like other transport proteins (e.g. ATP7A) and some lysosomal membrane proteins of unknown function (e.g. LEP100), it may be expressed on the plasma membrane under special metabolic circumstances (Hunziker and Geuze, 1996; Petris et al., 1996). Antibodies raised against the SASD gene product can be used to investigate its localization. Expression studies of mutated SASD gene constructs may contribute to our understanding of their effect on the protein level. The development of transgenic and knock-out mice may provide answers on the consequences of the mutations *in vivo*.

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## SUMMARY

The existence of specific lysosomal transport proteins became evident with the elucidation of two lysosomal storage diseases due to a transport defect, cystinosis and sialic acid storage disease. During the last 15 years, more than 20 lysosomal transport proteins for the transport of amino acids, sugars, nucleosides, inorganic ions, and vitamins have been characterized. In our laboratory, the acidic monosaccharide transporter, a neutral monosaccharide transporter and a chloride channel have earlier been described and characterized. At the beginning of the present studies, none of the lysosomal transporters had been purified and none of the corresponding genes had been cloned. Therefore, knowledge of their molecular structure was not available.

The experimental work in this thesis describes the first successful purification of a functionally active lysosomal transport protein, the sialic acid transporter, and the characterization of its functional properties. Additionally, it describes the characterization of a new lysosomal transporter involved in heavy metal metabolism.

A lysosomal proton-cotransporter with a specificity for sialic acid and glucuronic acid is defective in sialic acid storage disease (SASD). This autosomal recessive inherited disorder is characterized by lysosomal accumulation and excessive urinary excretion of free sialic acid. Abnormal sialic acid transport in lysosomes is the primary genetic defect in the two clinical variants of SASD: Salla disease and infantile sialic acid storage disease. The elucidation of the molecular structure and functional properties of this transport system is indispensable for further understanding of the molecular defect(s) in the clinical variants of SASD. A previously developed system for the solubilization and functional reconstitution of the sialic acid transporter provided the tool to start protein purification. The sialic acid transporter has now been purified to apparent homogeneity by a combination of hydroxyapatite, lectin, and ion exchange chromatography. A 57 kDa protein correlated with transport activity (**publication I**).

Kinetic studies on the functional properties of the purified protein showed that besides the acidic monosaccharides sialic acid and glucuronic acid, also iduronic acid is a substrate. These monocarboxylated sugars are physiological end products of the lysosomal degradation of glycoproteins, glycosaminoglycans or glycolipids. Comparison of the functional properties of our transporter with those of other monocarboxylate transporters revealed that at least *in vitro* the sialic acid transporter also translocates L-lactate (**publication I**). In subsequent kinetic studies we demonstrated that substrates of other anion transporters are also recognized: α-ketoglutarate, *para*-aminohippurate, valproate, salicylate, urate, methotrexate and folate (**publication II**). Among these, we demonstrated that the sialic acid transporter also translocates α-ketoglutarate across the lysosomal membrane. The structures of the recognized molecules are quite different, ranging from small aliphatic molecules (e.g. L-lactate) to more complex molecules (e.g. folate). The presence of one or two carboxylic groups seems to be a structural requirement

for recognition. On the basis of these functional studies, the sialic acid transporter can be classified within anion transporter families, including proton symporters, which show an overlap in substrate specificity. Those families are the sialate:$H^+$ symporters (SHS), the organic anion transporters (OAT), the metabolite:$H^+$ symporters (MHS), the monocarboxylate transporters (MCT), and the anion:cation symporters (ACS), which are all subfamilies of the major facilitator superfamily (MFS). Databases generated during the Human Genome Project contain many ESTs (expressed sequence tags) with homology to members of these transporter families. Candidate genes for SASD might be selected by mapping those ESTs and comparing their chromosomal localization to the linkage interval of SASD on chromosome 6q.

Hepatic lysosomes are able to sequester excess copper and to empty their contents into the bile. Therefore, besides their major function in degradation, lysosomes are also thought to play a role in the regulation of heavy metal metabolism. In **publication III**, we describe a new method to study copper transport. Transport studies using radioactive copper ($^{64}Cu$) are difficult to perform, because of its scarce availability and very short physical half life (12.8 hours). The existing copper loading test for one of the genetic diseases with a disturbed copper metabolism, Menkes disease, is therefore cumbersome. This test can only be performed in a few specialized centers in the world. We here developed a diagnostic loading test for Menkes disease by using radioactive silver ($^{110m}Ag^+$), which is commercially available and has a convenient physical half life of 250 days (**publication III**). These studies support the hypothesis that reduction of divalent to monovalent copper is an essential step preceding transport. The finding that radioactive silver can replace copper provided the opportunity to study lysosomal heavy metal (copper) transport in lysosomes. With the characterization of a heavy metal ion P-type ATPase, we provided direct evidence that lysosomes are able to take up and possibly sequester heavy metal ions (**publication IV**). The transporter showed specificity for silver, copper and cadmium ions. A possible role of this new lysosomal transporter in Wilson disease, a copper accumulation disorder, was excluded in the existing animal model.

## SAMENVATTING

Het bestaan van specifieke lysosomale transport eiwitten werd duidelijk door de opheldering van twee lysosomale stapelingsziekten, cystinosis en siaalzuur stapelingsziekte, die beiden worden veroorzaakt door een transport defect. In de afgelopen 15 jaar zijn er meer dan 20 lysosomale transport eiwitten gekarakteriseerd. Er bestaan transporters voor aminozuren, suikers, nucleosiden, anorganische ionen en vitaminen. Drie van deze transport eiwitten zijn in het verleden in ons laboratorium beschreven en gekarakteriseerd: een transporter voor zure monosacchariden, één voor neutrale monosacchariden en een chloride kanaal. Bij aanvang van de studies beschreven in dit proefschrift was geen enkele lysosomale transporter gezuiverd of gekloneerd, waardoor hun moleculaire structuur onbekend was.

Het experimentele werk beschreven in dit proefschrift omvat de eerste succesvolle zuivering van een functioneel actief lysosomaal transport eiwit, de siaalzuur transporter, en de karakterisering van zijn functionele eigenschappen. Daarnaast beschrijft het ook de karakterisering van een nieuw lysosomaal transport systeem, dat betrokken is bij het metabolisme van zware metalen.

Een lysosomale proton-cotransporter met een specificiteit voor siaalzuur en glucuronzuur is defect in siaalzuur stapelingsziekte (SASD). Deze autosomaal recessieve ziekte wordt gekenmerkt door een lysosomale stapeling en excessieve uitscheiding via de urine van vrij siaalzuur. Abnormaal siaalzuur transport in de lysosomen van patienten is het primaire genetische defect in de twee klinische varianten van SASD: de ziekte van Salla en de infantiele siaalzuur stapelingsziekte. Om de moleculaire defecten bij de klinische varianten van de siaalzuur stapelingsziekte beter te begrijpen, is opheldering van de moleculaire structuur en functionele eigenschappen van dit transport eiwit gewenst. Door de ontwikkeling van een systeem, waarbij de siaalzuur transporter uit het lysosomale membraan wordt geNxtraheerd en vervolgens functioneel wordt ingebouwd in een artificieel membraan, werd het mogelijk de zuivering van dit eiwit te starten. De siaalzuur transporter is nu in zuivere vorm verkregen door middel van een combinatie van hydroxyapatite, lectine, en ionen wisselaar chromatografie. De transport acitiviteit was gerelateerd aan de aanwezigheid van een eiwit met een molecuul gewicht van ongeveer 57 kDa (**publicatie I**). Kinetische studies omtrent de functionele eigenschappen van het gezuiverde eiwit lieten zien dat naast de zure monosacchariden siaalzuur en glucuronzuur, ook iduronzuur behoort tot de substraten van dit eiwit. Deze suikers met een monocarboxyl groep zijn fysiologische eindproducten van de lysosomale afbraak van glycoproteçnen, glycosaminoglycanen of glycolipiden. Vergelijking van de functionele eigenschappen van onze transporter met die van andere transporters voor monocarboxylaten liet zien dat de siaalzuur transporter *in vitro* ook L-lactaat transporteert (**publicatie I**). Uit vervolg onderzoek is gebleken dat ook substraten van andere anion transporters worden herkend door de siaalzuur transporter: α-ketoglutaraat, *para-*

aminohippuraat, valproaat, salicylaat, uraat, methotrexaat en folaat (**publicatie II**). Van deze moleculen wordt α-ketoglutaraat niet alleen herkend, maar ook getransporteerd. De structuren van de moleculen die worden herkend zijn zeer verschillend, uiteenlopend van kleine alifatische moleculen, zoals L-lactaat, tot meer complexe moleculen, zoals folaat. De aanwezigheid van één of twee carboxyl groepen in hun structuur lijkt een vereiste voor herkenning. Op basis van deze functionele studies kan de siaalzuur transporter worden ingedeeld bij anion transporter families, die proton symporters bevatten en een overlap hebben in substraat specificiteit. Deze families zijn de sialaat:$H^+$ (SHS) symporters, de organische anion transporters (OAT), the metaboliet:$H^+$ symporters (MHS), de anion:cation symporters (ACS) en de monocarboxylaat transporters (MCT), die allen subfamilies zijn van de "major facilitator superfamily" (MFS). Databanken die voortgekomen zijn uit het Humane Genoom Project bevatten vele ESTs ("expressed sequence tags"), die homologie hebben met leden van deze transporter families. Kandidaat genen voor SASD zijn die ESTs, die na bepaling van hun chromosomale localisatie in hetzelfde gebied gelegen zijn als het bekende locus voor SASD op chromosoom 6q14-q15.

Lysosomen in hepatocyten zijn in staat om koper op te nemen ten tijde van overvloed en hun inhoud te legen in de gal. Hierdoor wordt gedacht dat lysosomen, naast hun belangrijkste functie in afbraak, ook betrokken zijn bij de regulatie van het metabolisme van zware metalen. In **publicatie III**, beschrijven we een nieuwe methode om koper transport te kunnen bestuderen. Transport studies, waarin radioactief koper wordt gebruikt zijn ingewikkeld om uit te voeren, omdat dit materiaal moeilijk te verkrijgen is en een zeer korte halfwaarde tijd heeft (12.8 uur). Om die rede is de huidige diagnostische koper belastingstest voor één van de erfelijke ziekten met een verstoord koper metabolisme, de ziekte van Menkes, moeilijk in het gebruik. Deze test kan maar in een paar centra ter wereld worden uitgevoerd. Wij hebben nu een diagnostische belastingstest voor de ziekte van Menkes ontwikkeld waarbij we gebruik maken van radioactief zilver ($^{110m}$ Ag). Dit radioactief materiaal is commercieel verkrijgbaar en heeft een gunstige halfwaarde tijd van 250 dagen (**publicatie III**). Dit onderzoek liet tevens zien dat reductie van divalent in monovalent koper een belangrijke stap is voordat er transport kan plaatsvinden. Het feit dat radioactief zilver koper kan vervangen in transport studies, maakte het mogelijk om transport van zware metalen (koper) over het lysosomale membraan te bestuderen. Met de karakterisering van een P-type ATPase voor zware metaal ionen in het lysosomale membraan hebben we aangetoond dat lysosomen in staat zijn om zware metaal ionen op te nemen en eventueel op te slaan (**publicatie IV**). De transporter heeft een specificiteit voor zilver, koper en cadmium ionen. Een mogelijke rol voor deze nieuwe lysosomale transporter in Wilson disease, een koper stapelingsziekte, werd uitgesloten in het bestaande diermodel.

## ABBREVIATIONS

| | |
|---|---|
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| DEPC | Diethyl pyrocarbonate |
| ER | Endoplasmatic reticulum |
| EST | Expressed sequence tag |
| GlcA | Glucuronic acid |
| IdoA | Iduronic acid |
| ISSD | Infantile sialic acid storage disease |
| LAMP | Lysosome associated membrane glycoprotein |
| LEC | Long Evans Cinnamon |
| LIMP | Lysosome integral membrane glycoprotein |
| LPI | Lysinuric protein intolerance |
| MDCK | Madin-Darby canine kidney |
| MRI | Magnetic resonance imaging |
| NEM | *N*-acetylmaleimide |
| Neu5Ac | N-acetylneuraminic acid |
| NMR | Nuclear magnetic resonance |
| SASD | Sialic acid storage disease |
| TGN | *trans*-Golgi network |

## CURRICULUM VITAE

| | |
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Stellingen behorende bij het proefschrift

# Lysosomal membrane transport proteins and their significance in human genetic disease

1. Zonder transport staat alles stil.
   *Transport en Logistiek Nederland*
   *Dit proefschrift*

2. Functionele studies moeten niet worden onderschat bij het kloneren van ziektegenen.
   *Dit proefschrift*

3. Het toeschrijven van een functie aan een genprodukt alleen op basis van sequentie homologie dient met voorzichtigheid te geschieden.
   *Everett et al., (1997) Nat. Genet. 17:411-422*
   *Scott et al., (1999) Nat. Genet. 21:440-443*
   *Dit proefschrift*

4. Radioactief zilver kan radioactief koper vervangen in de diagnostische belastingstest voor de ziekte van Menkes.
   *Dit proefschrift*

5. Digitalisering zet gemakkelijk aan tot fraudering.

6. Het algemeen testen van jonge kinderen op dyslexie zou vroeg ingrijpen mogelijk maken en daarmee langdurige problemen van vele dyslectici doen verminderen.
   *Clayton, New Sci. (1999) 162:27-30*

7. Door de enorme groei van de wereldbevolking is het gebruik van genetisch gemanipuleerd voedsel onontkoombaar.

8. Bij promoveren spelen zowel intrinsieke motivatie (succes door volharding) als extrinsieke motivatie (succes door geluk) een belangrijke rol.
*v.Yperen en Diderich, Ned. Tijdschr. Psychol. (1998) 53:76-84*

9. Per definitie keer je altijd de helft van de wereld de rug toe.

10. De feminisatie van de wetenschap neemt toe naarmate men verder afdaalt langs de rangen.

11. Het schrijven van een proefschrift is een cognitieve afleiding voor een promovendus die "aan het lijnen is", waardoor overeten onvermijdelijk is.
*Boon et al., Br. J. Health Psychol. (1998) 3:27-40*
*Deze promovenda*

12. Een computer is een vriend: hij veroorzaakt vaak een gevecht, je kunt er om lachen en grienen, maar hij is stil en oprecht.

Adrie Havelaar Rotterdam, 21 juni 1999