

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.

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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.

2. Materials and methods

2.1. Materials

$^{110\text{m}}\text{Ag}$ was purchased from Amersham (specific activity of $1 \mu\text{Ci}/\mu\text{g}$ 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 membrane 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 $20000\times g$ 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 $8000\times g$ 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 $^{110\text{m}}\text{Ag}$ 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 $^{110\text{m}}\text{Ag}$ (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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Abbreviations: VacA, vacuolating cytotoxin A; TPA, 12-*O*-tetradecanoylphorbol-13-*O*-acetate; DMSO, dimethyl sulfoxide; NaBu, sodium butyrate; EGF, human epidermal growth factor

suspensions were pre-warmed at 37°C for 3 min. The uptake experiments at 37°C were started by adding a 5- μ l aliquot of 32 mM MgATP in 50 mM KHEPES, pH 7.4, to a 10- μ l aliquot of the ^{110m}Ag /GSH suspension and subsequently to a 25- μ l 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 μ l of ice-cold stop-solution (50 mM KHEPES, pH 7.4) and 100 μ l were immediately applied to a Sephadex G50 fine (Pharmacia LKB) column (Pasteur pipettes, 0.5 \times 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- μ l 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

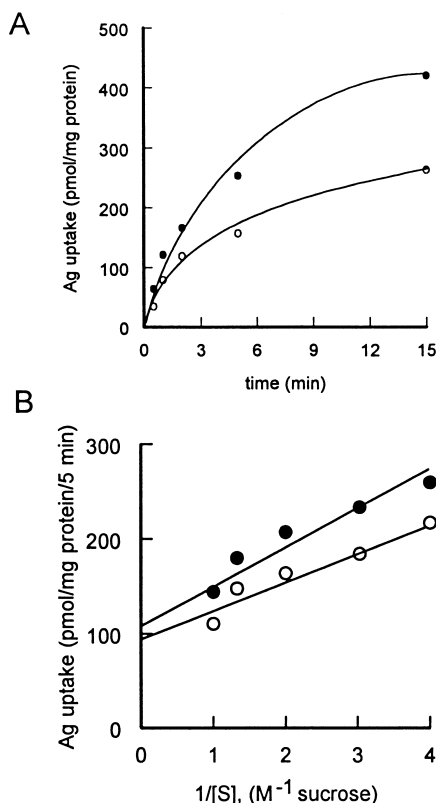


Fig. 1. A: ^{110m}Ag uptake in lysosomal membrane vesicles is stimulated by ATP. Membrane vesicles (100 μg of protein) were pre-incubated at 20°C for 10 min in medium containing 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 μM valinomycin, and 5 μM FCCP. Assays at 37°C were started by the addition of 7.5 μM ^{110m}Ag , 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.

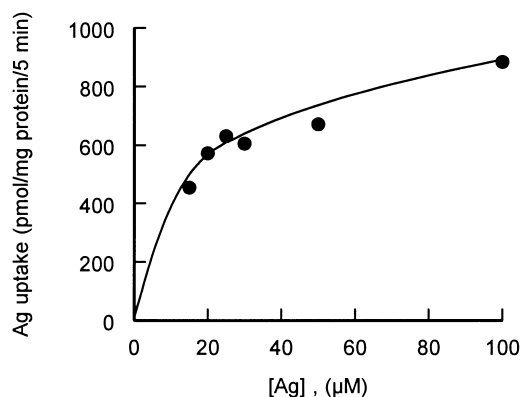


Fig. 2. Carrier mediated uptake of ^{110m}Ag into lysosomal membrane vesicles. Lysosomal membrane vesicles were pre-incubated as described in Fig. 1A. ^{110m}Ag uptake (5 min, 37°C) was measured in the presence of 4 mM ATP and increasing concentrations of Ag, and corrected for aspecific binding as measured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

lysosomal membrane vesicles. All experiments were performed in duplicate.

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

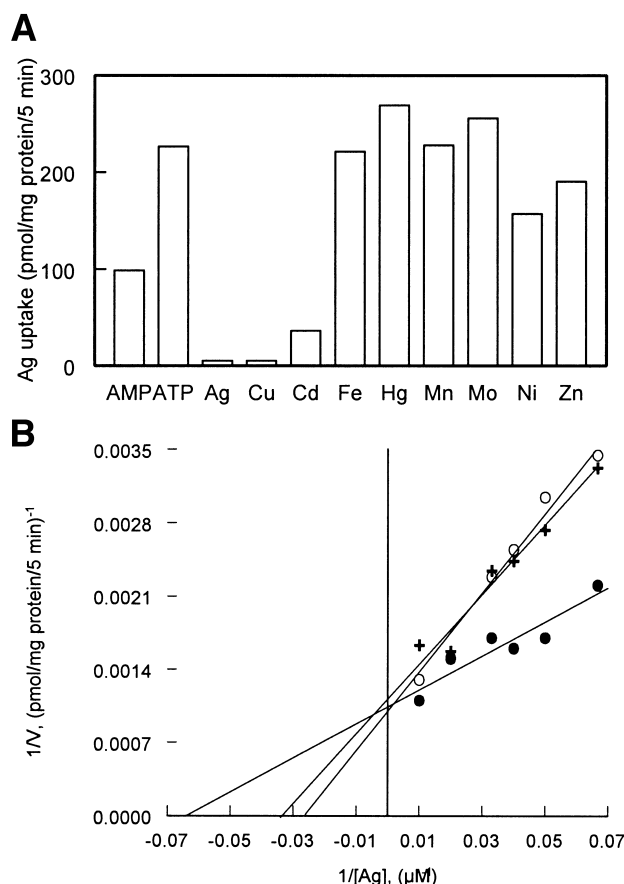


Fig. 3. A: *Cis*-inhibition of metal ions on the ATP stimulated uptake of ^{110m}Ag . Lysosomal membrane vesicles were incubated for 5 min at 37°C in the presence of 7.5 μM ^{110m}Ag , 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 μM valinomycin, 5 μM FCCP, 4 mM AMP or ATP and 100 μM of the indicated metal sulfates. Values are mean \pm 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: $K_i = K_m[I]/\{(-1/x) - K_i\}$ (K_i 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 \pm 0.7 | |
| Nucleotide | | |
| ATP | 145.8 \pm 18.6 | 158 |
| CTP | 148.2 \pm 0.6 | 160 |
| GTP | 115.4 \pm 24.8 | 125 |
| AMP | 83.7 \pm 6.8 | 91 |
| CMP | 89.4 \pm 4.9 | 97 |
| GMP | 78.1 \pm 21.4 | 85 |
| Non-hydrolyzable ATP-analogues | | |
| AMP-PCP | 89.6 \pm 12.0 | 97 |
| AMP-PNP | 77.6 \pm 14.4 | 84 |

^aNet uptake in the presence of 50 mM KHEPES, 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.

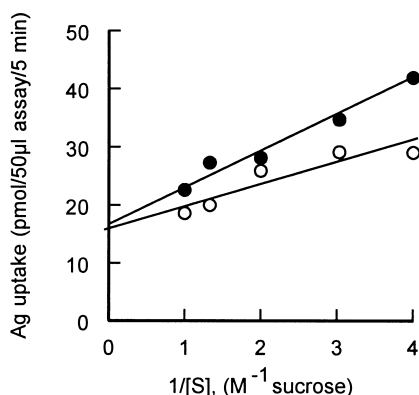


Fig. 4. Effect of increasing medium osmolarity by sucrose on Ag uptake in intact lysosomes. Percoll gradient isolated intact lysosomes were pre-incubated in 50 mM KHEPES, pH 7.4, 10 mM GSH, 5 μ M valinomycin, 5 μ M FCCP, and with 0.25–1 M sucrose for 1 h at 20°C. Lysosomes were then incubated for 5 min at 37°C with 15 μ M 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 \pm 4.2 | – | – |
| AMP | 4 | 178.5 \pm 20.1 | 58 | – |
| ATP+KNO ₃ | 50 | 313.8 \pm 28.5 | 102 | V-type ATPase |
| ATP+N-ethylmaleimide | 1 | 335.5 \pm 53.9 | 109 | V-type ATPase |
| ATP+bafilomycin A1 | 0.001 | 283.9 \pm 9.3 | 93 | V-type ATPase |
| ATP+NaN ₃ | 5 | 333.6 \pm 30.4 | 109 | F-type ATPase |
| ATP+VO ₄ ³⁻ | 0.1 | 296.7 \pm 30.2 | 97 | P-type ATPase |
| ATP+DIDS | 1 | 156.3 \pm 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 μ M CuSO₄. 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. $\text{GS}^-\text{..}^+\text{Zn}^+\text{..}^-\text{SG}$) [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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