Mercuric Conjugates of Cysteine Are Transported by the Amino Acid Transporter System $b^{0,+}$: Implications of Molecular Mimicry

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Abstract. Humans and other mammals continue to be exposed to various forms of mercury in the environment. The kidneys, specifically the epithelial cells lining the proximal tubules, are the primary targets where mercuric ions accumulate and exert their toxic effects. Although the actual mechanisms involved in the transport of mercuric ions along the proximal tubule have not been defined, current evidence implicates mercuric conjugates of cysteine, primarily 2-amino-3-(2-amino-2-carboxyethylsulfanylmercuricsulfanyl)propionic acid (Cys-*S*-Hg-*S*-Cys), as the most likely transportable species of inorganic mercury (Hg^{2+}). Because Cys-*S*-Hg-*S*-Cys and the amino acid cystine (Cys-*S*-*S*-Cys) are structurally similar, it was hypothesized that Cys-*S*-Hg-*S*-Cys might act as a molecular mimic of cystine at one or more of the amino acid transporters involved in the luminal absorption of this amino acid. One such candidate is the Na^+-independent heterodimeric transporter system $b^{0,+}$. Therefore, the transport of Cys-*S*-Hg-*S*-Cys and cystine was studied in MDCK II cells that were or were not stably transfected with $b^{0,+}$AT-rBAT. Transport of Cys-*S*-Hg-*S*-Cys and cystine across the luminal plasma membrane was similar in the transfected cells, indicating that Cys-*S*-Hg-*S*-Cys can behave as a molecular mimic of cystine at the site of system $b^{0,+}$. Moreover, only the $b^{0,+}$AT-rBAT transfectants became selectively intoxicated during exposure to Cys-*S*-Hg-*S*-Cys. These findings indicate that system $b^{0,+}$ likely contributes to the nephropathy induced by Hg^{2+} *in vivo*. These data represent the first direct molecular evidence for the participation of a specific transporter in the luminal uptake of a large divalent metal cation in proximal tubular cells.

A large body of evidence indicates that the epithelial cells lining the convoluted and straight segments of the proximal tubule are the primary sites where inorganic mercury (Hg^{2+}) is taken up and accumulated *in vivo* (1,2). Although the actual mechanisms by which mercuric ions are taken up by these cells are not well defined, a number of recent *in vivo* findings indicate that the uptake of Hg^{2+} at the luminal membrane of proximal tubular cells is dependent on the activities of the brush border enzymes γ-glutamyltransferase (3–8) and cysteinylglycinase (8). It seems that mercuric conjugates of GSH, while in the lumen of the proximal tubule, are degraded sequentially by these enzymes to yield a cysteine-*S*-conjugate of mercury, primarily 2-amino-3-(2-amino-2-carboxyethylsulfanylmercuricsulfanyl)propionic acid (Cys-*S*-Hg-*S*-Cys) (2). This conjugate is thought to be the principal species of Hg^{2+} taken up at the luminal plasma membrane of proximal tubular epithelial cells (3–8).

Recent studies using brush border membrane vesicles (9) and isolated perfused proximal tubular segments (8,10) indicate that Cys-*S*-Hg-*S*-Cys is indeed transported across the luminal membrane of proximal tubular epithelial cells. Moreover, competitive inhibition experiments using isolated perfused proximal tubular segments have implicated amino acid transporters as the main carrier proteins involved in the absorption of Cys-*S*-Hg-*S*-Cys from the proximal tubular lumen (8,10).

Interestingly, the molecular structure of Cys-*S*-Hg-*S*-Cys is very similar to that of the amino acid cystine (Cys-*S*-*S*-Cys). This structural similarity, and the fact that amino acid transporters have been implicated in the luminal uptake of Hg^{2+}, led us to hypothesize that Cys-*S*-Hg-*S*-Cys can act as a "molecular mimic," or homolog of cystine, and can be taken up into proximal tubular cells via one or more of the luminal transporters of cystine. Current evidence indicates that both Na^+-dependent and Na^+-independent mechanisms are involved in the absorptive luminal transport of Cys-*S*-Hg-*S*-Cys in the proximal tubule (10). Moreover, preliminary data indicate that the Na^+-dependent component of Cys-*S*-Hg-*S*-Cys transport may be mediated by system(s) ASC and/or $B^{0,+}$, whereas a likely candidate for the Na^+-independent uptake of Cys-*S*-Hg-*S*-Cys is system $b^{0,+}$ (8).

System $b^{0,+}$ is a heterodimeric amino acid transporter that is composed of a light chain, $b^{0,+}$AT, and a heavy chain, rBAT

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(11,12). More importantly, this amino acid transporter has a high affinity for cystine (11,12) and, in the kidneys, is localized exclusively in the luminal plasma membrane of the target epithelial cells, *i.e.*, proximal tubular cells (13–15). On the basis of these characteristics, we hypothesize that system $b^{0,+}$ is capable of transporting mercuric ions, in the form of Cys-*S*-Hg-*S*-Cys, into the intracellular compartment of renal epithelial cells.

To test this hypothesis, we studied the kinetics and characteristics of the transport of Cys-*S*-Hg-*S*-Cys in MDCK II cells that were or were not stably transfected with system $b^{0,+}$. These cells represent an immortalized line of renal epithelial cells derived from the distal nephron of a dog and were used for these experiments because they do not normally express $b^{0,+}$AT or rBAT. Biochemical data from this study clearly indicate that Hg^{2+}, in the form of Cys-*S*-Hg-*S*-Cys, is a transportable substrate of system $b^{0,+}$. Furthermore, our toxicity data indicate that this transporter may contribute to the proximal tubular nephropathy induced by Hg^{2+} *in vivo*. This study is the first to provide direct molecular evidence that a specific amino acid transport system, namely, system $b^{0,+}$, is involved in the absorptive transport of a biologically relevant molecular species of Hg^{2+} (*i.e.*, Cys-*S*-Hg-*S*-Cys).

Materials and Methods

Tissue Culture and Stable Transfection

The details of the transfection of MDCK II cells with mouse $b^{0,+}$AT and human rBAT ($b^{0,+}$AT-rBAT transfectants) were described previously (16). Transfectants were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA) supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1% essential amino acids, 200 μg/ml geneticin, and 150 μg/ml hygromycin B (Invitrogen, Carlsbad, CA), as described previously (16). Wild-type cells were cultured in the same medium without geneticin or hygromycin B.

Reverse Transcription-PCR

Total RNA was isolated from the cells with the use of TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription-PCR analyses were performed with a GeneAmp kit (Applied Biosystems, Foster City, CA). The presence of mouse $b^{0,+}$AT and human rBAT was assayed with primers specific for each subunit, yielding 589-bp and 600-bp fragments, respectively (16).

Laser Scanning Confocal Microscopy

Immunohistochemical experiments were performed as described previously (16). Briefly, cells were incubated with a polyclonal antibody against mouse $b^{0,+}$AT (16) (1:500), followed by incubation with 1 μg/ml Texas Red-labeled anti-rabbit IgG Fab fragments (Rockland, Gilbertsville, PA). Cells were then incubated with a polyclonal antibody against human rBAT (16) (1:50), followed by incubation with FITC-labeled anti-rabbit IgG antibody (Sigma Chemical Co., St. Louis, MO). Analyses of the cell monolayers were performed with a Leica laser scanning confocal microscope (TCSSP, Wetzlar, Germany).

Manufacture of $^{203}Hg^{2+}$

Three milligrams of mercuric oxide were doubly sealed in quartz tubing with an acetylene torch. The relative concentrations of the various isotopes of mercury in the sample of mercuric oxide were as follows: $<0.05\%$ ^{196}Hg, 1.5% ^{198}Hg, 2.82% ^{199}Hg, 4.24% ^{200}Hg, 3.11% ^{201}Hg, 86.99% ^{202}Hg (target), and 1.34% ^{204}Hg. The encapsulated mercury was irradiated via neutron activation for 4 wk, at the Missouri University Research Reactor facility. After irradiation, the product was placed in protected storage for 10 d, to facilitate the decay of the newly formed $^{197}Hg^{2+}$. After removal of the outer quartz tube, the inner tube was crushed and rinsed with four 50-μl washes of 1 N HCl. All rinses were placed in a single polypropylene vial. To determine the exact solid content of mercury, a sample of the solution was subjected to plasma-coupled elemental mass spectrometry. The radioactivity of the solution was determined with a Perkin Elmer Wallac Wizard 3 automatic gamma counter (Perkin Elmer, Gaithersburg, MD). The specific activities of the ^{203}Hg ranged from 6 to 12 mCi/mg (17).

Evaluation of Transport

Uptake measurements were performed as described previously, with minor changes (18,19). Wild-type and transfected MDCK cells were seeded in 24-well plates, at a density of 0.2×10^6 cells/well, and were cultured for 24 h before the experiments. Transfectants were cultured in the presence of 1 μM dexamethasone, to induce the expression of rBAT (16). At the beginning of each experiment, culture medium was removed and cells were washed with warm uptake buffer (25 mM Hepes, 140 mM Tris, *N*-methyl-D-glucamine chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, pH 7.5). Uptake was initiated with the addition of 250 μl of uptake buffer containing radiolabeled substrates. Cells were incubated for 30 min at 37°C, unless otherwise stated. Uptake was terminated with the aspiration of radiolabeled compounds, followed by the addition of ice-cold uptake buffer containing 1 mM sodium 2,3-dimercaptopropane-1-sulfonate (Sigma), a well known mercury chelator (2). Cells were washed twice with 2,3-dimercaptopropane-1-sulfonate and solubilized with 1% SDS in 0.2 N NaOH, and the radioactivity in the cells was determined by liquid scintillation counting.

To fully characterize the transport processes in MDCK II cells, the uptake of ^{35}S cystine or Hg^{2+}, as a conjugate of cysteine (Cys-*S*-Hg-*S*-Cys), was measured under various conditions. Cys-*S*-^{203}Hg-*S*-Cys was obtained by incubating 5 μM $HgCl_2$, containing $^{203}Hg^{2+}$, with 20 μM cysteine for 10 min at room temperature. This ratio of cysteine to Hg^{2+} ensures that each mercuric ion in solution bonds to the sulfur atoms of two molecules of cysteine, in a linear II coordinate covalent manner. The mercuric conjugates of low-molecular weight thiols formed under these conditions have been demonstrated to be thermodynamically stable at pH 1 to 14 (20).

Time-course experiments were performed in which cells were incubated with either 5 μM cystine (containing ^{35}S) or 5 μM Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) for various periods ranging from 5 to 90 min. The saturation kinetics of the transport processes were analyzed by incubating cells with ^{35}S cystine (Amersham, Piscataway, NJ) or Cys-*S*-^{203}Hg-*S*-Cys, for 15 min at 37°C, in the presence of unlabeled cystine (25, 50, 75, 100, 250, 500, or 750 μM) or unlabeled Cys-*S*-Hg-*S*-Cys (0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, or 10 μM), respectively. The temperature dependence of the uptake of cystine (containing ^{35}S) and Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) was assessed by analyzing the saturation kinetics for transport of the substrates at 4°C and 37°C.

Substrate specificity was assessed by incubating cells with cystine (containing ^{35}S) or Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$), for 30 min at 37°C, in the presence of amino acids that are substrates of system $b^{0,+}$ (arginine, leucine, lysine, histidine, phenylalanine, glycine, or

cystine) or amino acids that are not transportable substrates of system $b^{0,+}$ (glutamate or aspartate). With the exception of cystine, all amino acids were used at a concentration of 3 mM. Because of low solubility, the highest attainable concentration of cystine was 1 mM.

In addition, 5 μM Hg^{2+} (as mercuric chloride, $HgCl_2$) was presented to the cells, for 30 min at 37°C, as a conjugate of cysteinylglycine (CysGly), *N*-acetylcysteine (NAC), or GSH. These conjugates were generated as described above for the generation of Cys-*S*-Hg-*S*-Cys. NAC-*S*-Hg-*S*-NAC was used as a negative control, because it is a highly polar molecule that is not taken up at the luminal plasma membrane of any renal epithelial cell. Mercuric conjugates of CysGly and GSH were used in this study because they are present in the proximal tubule lumen and are considered to be precursors of Cys-*S*-Hg-*S*-Cys.

Efflux Assays

Assays measuring the efflux of cystine were performed by preexposing both cell types to 1 mM cystine (containing ^{35}S) for 10 min at 37°C, followed by a 1-min incubation with 1 mM unlabeled cystine, Cys-*S*-Hg-*S*-Cys, glutamate, or arginine or uptake buffer. The extracellular fluid was then placed in scintillation vials for counting. Cells were washed twice with ice-cold buffer and solubilized with 1% SDS/0.2 N NaOH. The content of ^{35}S in the extracellular fluid and in the cells were determined with liquid scintillation counting.

Assessment of Cellular Viability

The toxicologic effects of $HgCl_2$, Cys-*S*-Hg-*S*-Cys, and GSH-*S*-Hg-*S*-GSH were measured with a methylthiazoletetrazolium assay, as described previously (17). Wild-type and transfected MDCK cells were seeded at a density of 0.2×10^6 cells/ml in 96-well culture dishes (200 μl/well). Cells were cultured for 24 h, washed twice with warm uptake buffer, and then were treated with $HgCl_2$, (1, 5, 10, 15, or 25 μM), Cys-*S*-Hg-*S*-Cys (100, 250, 500, 750, or 1000 μM), or GSH-*S*-Hg-*S*-GSH (100, 250, 500, 750, or 1000 μM) for 24 h at 37°C, in a humidified atmosphere of 5% CO_2. Cells were then washed twice with warm uptake buffer and incubated with 0.5 mg/ml methylthiazoletetrazolium for 2 h at 37°C, in a humidified atmosphere of 5% CO_2. After this incubation, solubilization buffer (10% Triton X-100 and 0.1 N HCl in isopropyl alcohol) was added to each well and the mixture was allowed to incubate for 16 h at room temperature. Each plate was read at 595 nm with a Titertek Multiskan MKII plate reader (Fisher Scientific, Suwanee, GA).

Data Analyses

All experiments were performed at least twice, with quadruplicate measurements for each condition. Data for each assessed parameter were analyzed first with the Kolmogorov-Smirnov test for normality and then with the Levene test for homogeneity of variances. The data were then analyzed with two-way ANOVA. Tukey's multiple-comparison procedure was used to assess differences among the means. Data expressed as a percentage of a total were normalized with the arc sine transformation before any parametric statistical analysis. This transformation calculates the arc sine of the square root of the decimal fraction of the percentage value. A P value of <0.05 was considered statistically significant.

Results

Expression of System $b^{0,+}$ in Cultured MDCK II Cells

Reverse transcription-PCR and laser scanning confocal microscopic analyses confirmed the expression of $b^{0,+}$AT and rBAT in the transfected MDCK II cells (Figure 1). Reverse transcription-PCR products of the expected sizes were obtained for mouse b^{0+}AT (589 bp) and human rBAT (600 bp). Wild-type cells did not express either subunit. Laser scanning confocal microscopic analyses of the transfectants demonstrated that mouse b^{0+}AT and human rBAT colocalized on the apical plasma membrane, as evidenced by the presence of yellow fluorescence (Figure 2A). Omission of the primary antibody against human rBAT served as a negative control experiment (Figure 2B).

Transport of Cystine and Hg^{2+} (as Cys-S-Hg-S-Cys)

Time-course analyses demonstrated that the uptake of cystine (Figure 3A) and Hg^{2+}, as Cys-*S*-Hg-*S*-Cys (Figure 3B), increased in both wild-type and transfected cells with time. The levels of uptake of both compounds were significantly greater in the transfectants at almost every time point studied.

The saturation kinetics for the uptake of cystine (Figure 4A) and Hg^{2+}, as Cys-*S*-Hg-*S*-Cys (Figure 4B), were analyzed in $b^{0,+}$AT-rBAT-transfected cells. When cystine was used as the substrate, the maximal velocity (V_{max}) of transport increased from 0.14 ± 0.08 nmol/mg protein per min in wild-type cells to 2.5 ± 0.5 nmol/mg protein per min in the transfectants. The

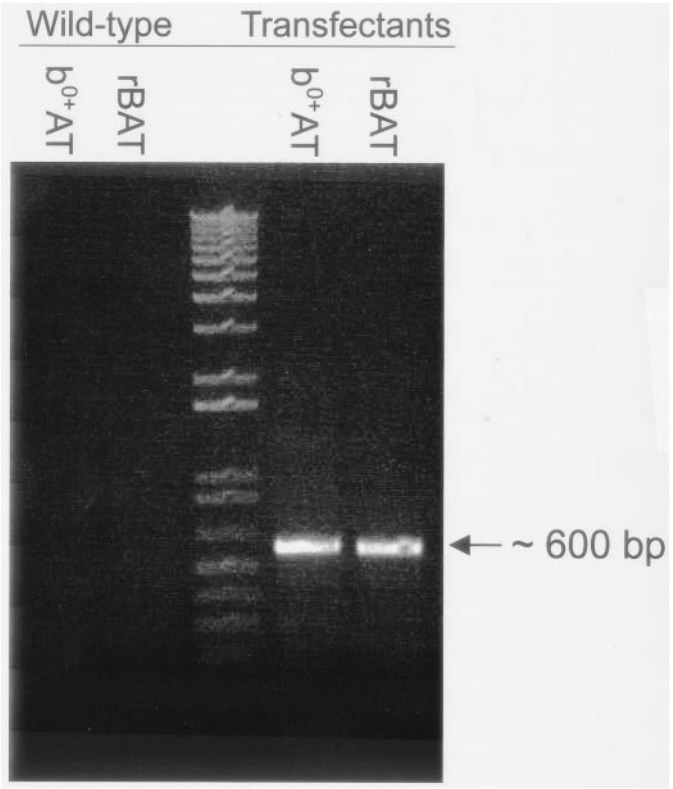

Figure 1. Agarose gel showing reverse transcription-PCR analysis of the steady-state expression of mRNA encoding mouse $b^{0,+}$AT and human rBAT in wild-type and $b^{0,+}$AT-rBAT-transfected MDCK II cells. The expected sizes of the reverse transcription-PCR products, as predicted from the positions of the primers, were 589 bp for mouse $b^{0,+}$AT and 600 bp for human rBAT. Transcripts for mouse $b^{0,+}$AT and human rBAT were not detected in wild-type cells.

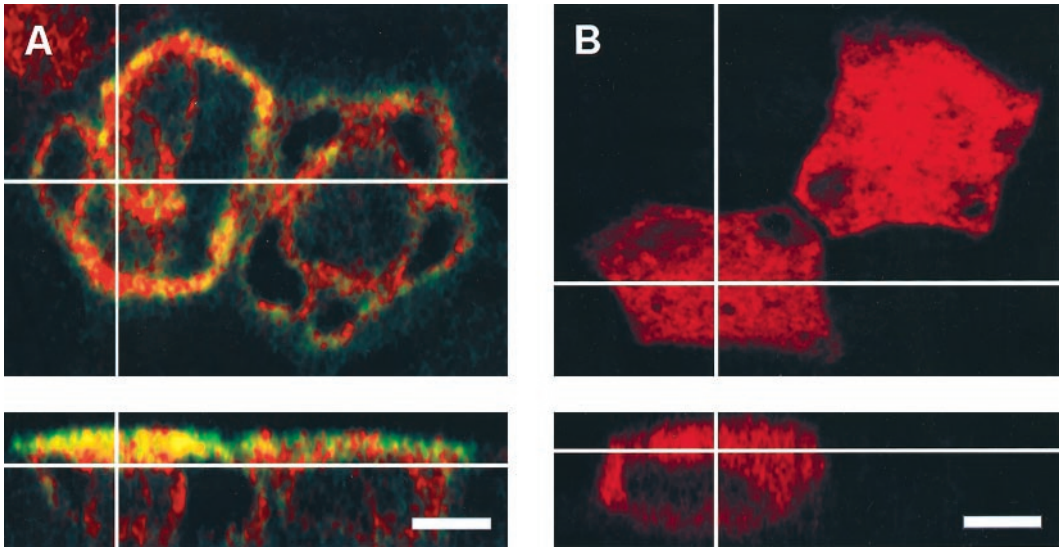

Figure 2. Laser scanning confocal microscopic images of $b^{0,+}$AT-rBAT-transfected MDCK II cells labeled with antibodies to mouse $b^{0,+}$AT and human rBAT, followed by incubation with Texas Red-labeled and FITC-labeled secondary antibodies, respectively. Upper panels, images of cells in a horizontal (*xy*) plane; lower panels, images of cells in a vertical (*zy*) plane. (A) Colocalization of mouse $b^{0,+}$AT and human rBAT on the plasma membranes of transfected cells. The distribution of mouse $b^{0,+}$AT is represented by red fluorescence, and the distribution of human rBAT is represented by green fluorescence. The yellow fluorescence represents the colocalization of these two proteins. The image in the vertical plane demonstrates the colocalization of these proteins on the apical plasma membrane of these cells. (B) Control experiment in which the primary antibody against human rBAT was omitted. Scale bars = 5 μm.

Michaelis-Menton constant (K_m) was calculated to be 0.12 ± 0.13 mM in wild-type cells and 0.67 ± 0.2 mM in the transfectants.

The uptake of Hg^{2+}, as Cys-*S*-Hg-*S*-Cys, was significantly greater in $b^{0,+}$AT-rBAT transfectants than in wild-type cells. The estimated V_{max} of transport increased from 5.1 ± 0.9 pmol/mg protein per min in wild-type cells to 8.8 ± 0.8 pmol/mg protein per min in the transfectants. The K_m was calculated to be 69.9 ± 12.1 μM in wild-type cells and 36.3 ± 7.6 μM in the transfectants.

The temperature dependence of system $b^{0,+}$ activity was measured by analyzing the saturation kinetics for the uptake of cystine (Figure 5A) and Hg^{2+}, as Cys-*S*-Hg-*S*-Cys (Figure 5B), in both cell types at 4°C and 37°C. The uptake of both substrates at 37°C was significantly greater in the transfectants than in corresponding groups of wild-type cells. When the experimental temperature was maintained at 4°C, there were no significant differences in the accumulation of cystine or Cys-*S*-Hg-*S*-Cys in corresponding groups of transfected and wild-type cells. Moreover, the amounts of substrate associated with either cell type at 4°C were similar to those associated with wild-type cells at 37°C.

The substrate specificity for the transport of cystine (Figure 6A) and Hg^{2+}, as Cys-*S*-Hg-*S*-Cys (Figure 6B), was measured in wild-type cells and $b^{0,+}$AT-rBAT transfectants. The uptake of cystine in the transfectants was significantly reduced in the presence of unlabeled cystine, cysteine, arginine, leucine, histidine, lysine, phenylalanine, or cycloleucine. The amino acids glutamate and aspartate, which are not substrates of system $b^{0,+}$, did not significantly alter the transport of cystine. With the exception of cells treated with cysteine or glutamate, there were no significant differences in the uptake of cystine among the groups of wild-type cells studied. This pattern of amino acid inhibition is in agreement with that demonstrated previously (16,21,22).

The pattern of substrate specificity for the transport of Cys-*S*-Hg-*S*-Cys was similar to that for cystine (Figure 6B). The uptake of Cys-*S*-Hg-*S*-Cys in the transfectants was significantly reduced by arginine, leucine, histidine, lysine, phenylalanine, and cycloleucine. The amino acids glutamate and aspartate did not significantly alter the transport of Cys-*S*-Hg-*S*-Cys. In wild-type cells, the presence of additional amino acids, with the exception of cysteine, did not significantly alter the transport of Cys-*S*-Hg-*S*-Cys. Interestingly, incubation with unlabeled cystine resulted in a twofold increase in the uptake of Cys-Hg-Cys in the transfectants. This stimulation was proportional to the concentration of cystine used (Figure 7A) and was independent of the period of incubation (data not shown). When the uptake of cystine (containing ^{35}S) was measured in the presence of excess Cys-*S*-Hg-*S*-Cys, transport in the transfectants was significantly inhibited (Figure 7B).

In a control experiment, the uptake of [^{3}H]arginine and [^{3}H]lysine, which are both substrates for system $b^{0,+}$, was measured in the presence and absence of unlabeled cystine. Exposure to cystine inhibited the transport of both lysine and arginine in the transfected cells but did not significantly alter the uptake of these amino acids in wild-type cells (data not shown).

Efflux Assays

Efflux assays were performed with wild-type cells and $b^{0,+}$AT-rBAT transfectants, to determine whether unlabeled

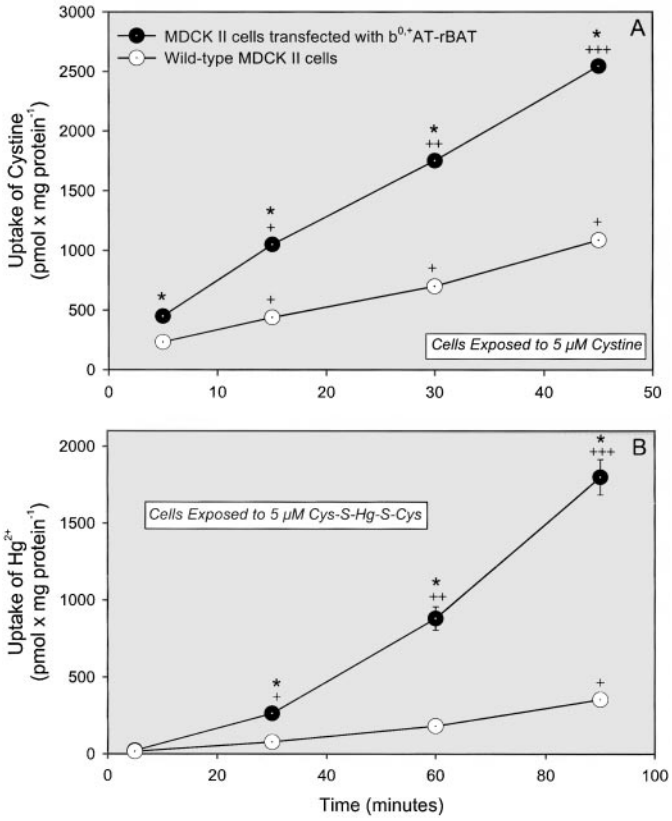

Figure 3. Time course of uptake of 5 μM cystine (containing [^{35}S]cystine) (A) or 5 μM inorganic mercury, as the mercuric conjugate of cysteine, 2-amino-3-(2-amino-2-carboxyethylsulfanylmercuricsulfanyl)propionic acid (Cys-*S*-Hg-*S*-Cys) (containing $^{203}Hg^{2+}$) (B), in wild-type and $b^{0,+}$AT-rBAT-transfected cells. Uptake was performed at 37°C for times ranging from 5 to 90 min. Samples were obtained for estimation at the indicated times. Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the same cell type at the initial time point; ++, significantly different from the mean for the same cell type at each of the two preceding time points; +++, significantly different from the mean for the same cell type at each of the three preceding time points.

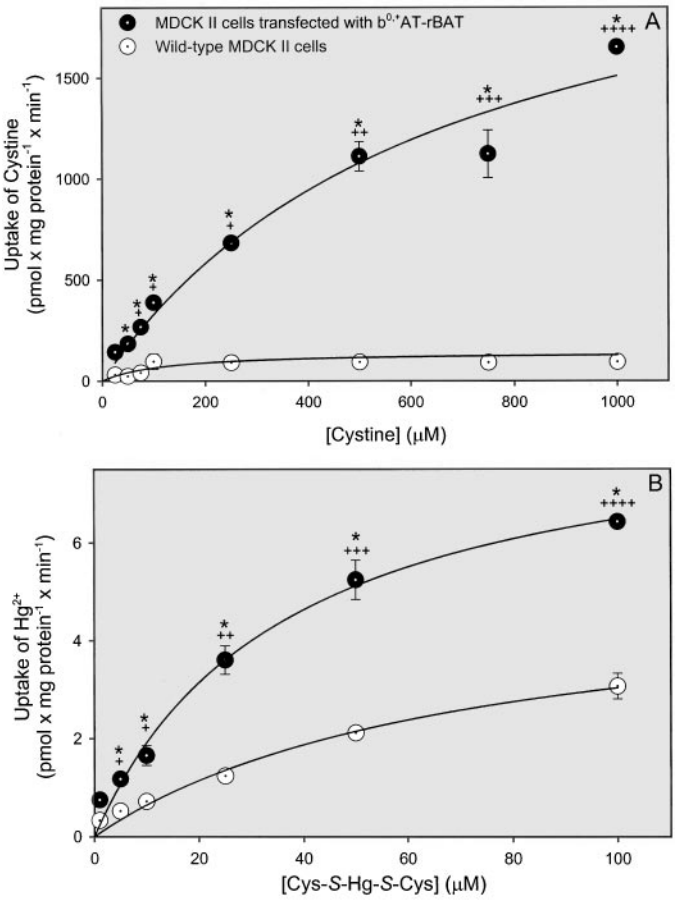

Figure 4. Saturation kinetics for the transport of cystine or inorganic mercury (Hg^{2+}), as the mercuric conjugate of cysteine, Cys-*S*-Hg-*S*-Cys, in wild-type and $b^{0,+}$AT-rBAT-transfected MDCK II cells. Cells were incubated for 30 min at 37°C with either 5 μM cystine (containing [^{35}S]cystine) (A) or 5 μM Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) (B), in the presence of unlabeled cystine (25 to 750 μM) or Cys-*S*-Hg-*S*-Cys (1 to 100 μM), respectively. Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the same cell type at the initial concentration; ++, significantly different from the mean for the same cell type at each of the preceding concentrations; +++, significantly different from the mean for the same cell type at each of the preceding concentrations; ++++, significantly different from the mean for the same cell type at each of the preceding concentrations.

Cys-*S*-Hg-*S*-Cys, cystine, arginine, or lysine was able to stimulate the efflux of [^{35}S]cystine/[^{35}S]cysteine (some of the cystine may be reduced intracellularly) (Figure 8A). The efflux of ^{35}S from the transfectants was greatest when cells were treated with Cys-*S*-Hg-*S*-Cys (123.2 ± 8.0 nmol/mg per min). This efflux was significantly greater than that in corresponding cells exposed to cystine (89.6 ± 2.2 nmol/mg per min) or arginine (86.4 ± 1.6 nmol/mg per min). The efflux of ^{35}S was even lower when the extracellular fluid consisted of uptake buffer alone (37.8 ± 4.5 nmol/mg per min) or contained glutamate (26.0 ± 1.6 nmol/mg per min). The mean levels of uptake did not differ significantly between the buffer and glutamate groups. When the cellular content of ^{35}S were measured, the pattern of uptake corresponded inversely to the pattern of efflux described above, *i.e.*, with greater efflux, lower cellular content of ^{35}S was observed (Figure 8B). There were no significant differences in the efflux or cellular contents of ^{35}S among the treatment groups of wild-type cells.

Transport of Mercuric Conjugates of Thiol-Containing Biologic Molecules

The uptake of Hg^{2+} as a mercuric conjugate of cysteine (Cys-*S*-Hg-*S*-Cys), NAC (NAC-*S*-Hg-*S*-NAC), CysGly (CysGly-*S*-Hg-*S*-CysGly), or glutathione (GSH-*S*-Hg-*S*-GSH) was measured in wild-type cells and transfectants (Figure 9). There

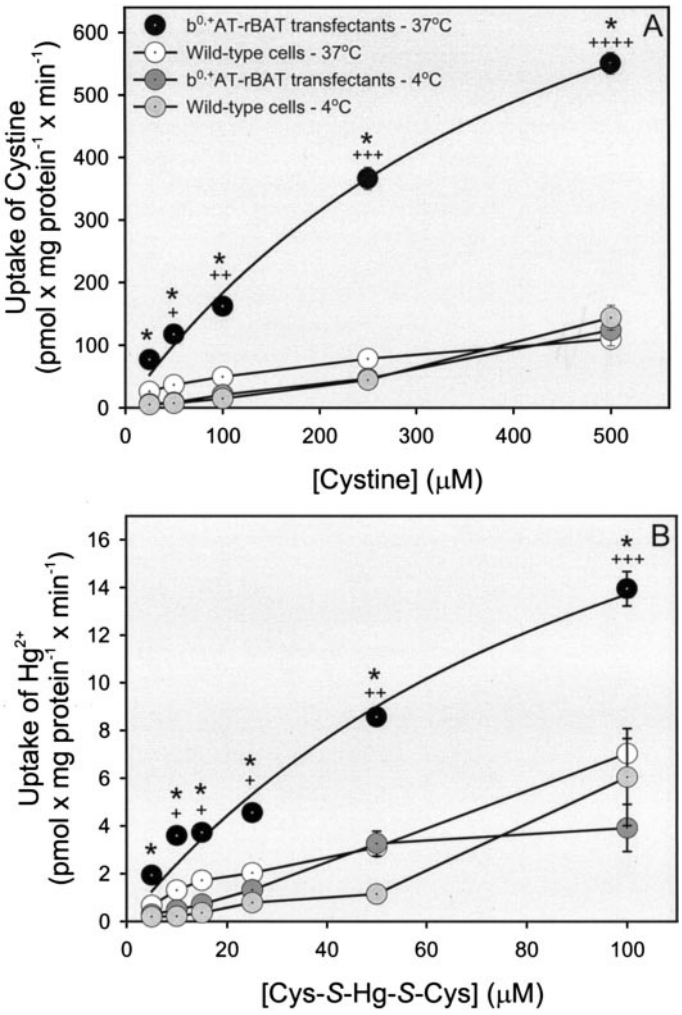

Figure 5. Temperature dependence of uptake of 5 μM cystine (containing [^{35}S]cystine) (A) or 5 μM inorganic mercury, as the mercuric conjugate of cysteine, Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) (B), in wild-type and $b^{0,+}$AT-rBAT-transfected cells. Uptake was measured in the presence of unlabeled cystine (25 to 500 μM) or Cys-*S*-Hg-*S*-Cys (1 to 100 μM), respectively, at 37°C or 4°C. Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding groups of wild-type cells at 4°C and 37°C and significantly different from the mean for the corresponding group of transfected cells at 4°C; +, significantly different from the mean for the transfectants at the initial concentration at 37°C; ++, significantly different from the mean for the transfectants at each of the preceding concentrations at 37°C; +++, significantly different from the mean for the transfectants at each of the preceding concentrations at 37°C; ++++, significantly different from the mean for the transfectants at each of the preceding concentrations at 37°C.

were no significant differences in the uptake of Hg^{2+} between corresponding groups of wild-type and transfected MDCK II cells when they were exposed to NAC-*S*-Hg-*S*-NAC, CysGly-*S*-Hg-*S*-CysGly, or GSH-*S*-Hg-*S*-GSH. In contrast, when the transfectants were exposed to Cys-*S*-Hg-*S*-Cys, the uptake of Hg^{2+} was at least twofold greater than that in the corresponding group of wild-type cells.

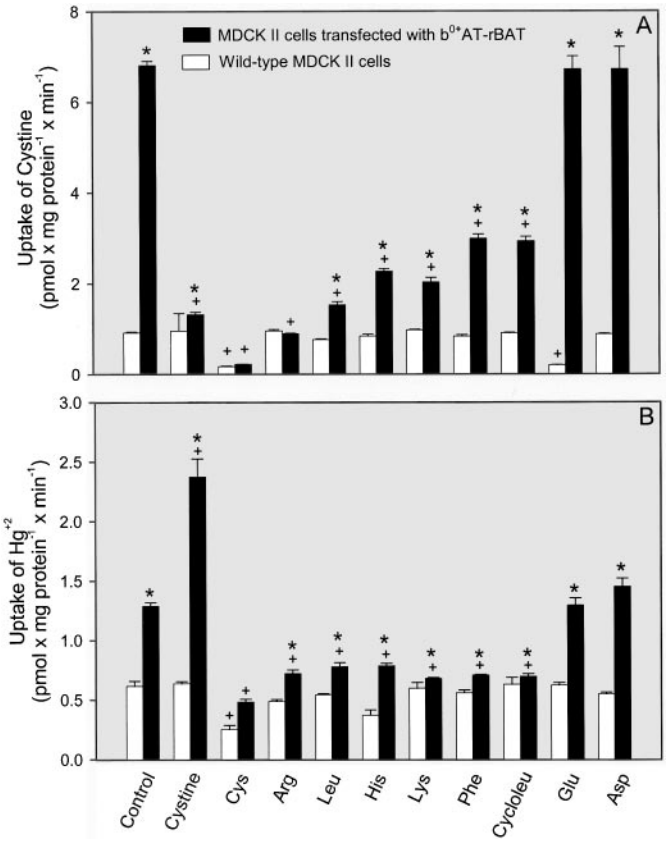

Figure 6. Substrate specificity analyses of the uptake of cystine (containing [^{35}S]cystine) (A) or inorganic mercury (Hg^{2+}), as the conjugate of cysteine, Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) (B), in wild-type and $b^{0,+}$AT-rBAT-transfected MDCK II cells. Cells were incubated for 30 min at 37°C with either 5 μM cystine (containing [^{35}S]cystine) or 5 μM Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$), in the presence of various unlabeled amino acids (at 3 mM, except for unlabeled cystine at 1 mM). Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the control group of the corresponding cell type.

Cellular Viability Assays

To determine the relationship between cellular transport and intoxication, the viability of wild-type and transfected cells exposed to various species of Hg^{2+} was assessed. After a 24-h exposure to 100 μM Cys-*S*-Hg-*S*-Cys, the viability of the $b^{0,+}$AT-rBAT transfectants was reduced by 12%, whereas exposure to 1 mM Cys-*S*-Hg-*S*-Cys decreased the viability of those cells by 78% (Figure 10A). Cellular viability was not significantly affected in corresponding wild-type cells. Treatment with 5 μM Cys-*S*-Hg-*S*-Cys for 30 min (the experimental conditions used to study various characteristics of transport) did not significantly reduce the viability of either cell type (data not shown). Incubation with GSH-*S*-Hg-*S*-GSH also did not alter the viability of the cells at any concentration studied (Figure 10B). Because $HgCl_2$ has been demonstrated to induce cell death in any cultured cell, it was used as a positive control. The viability of both cell types began to decline with 1 μM

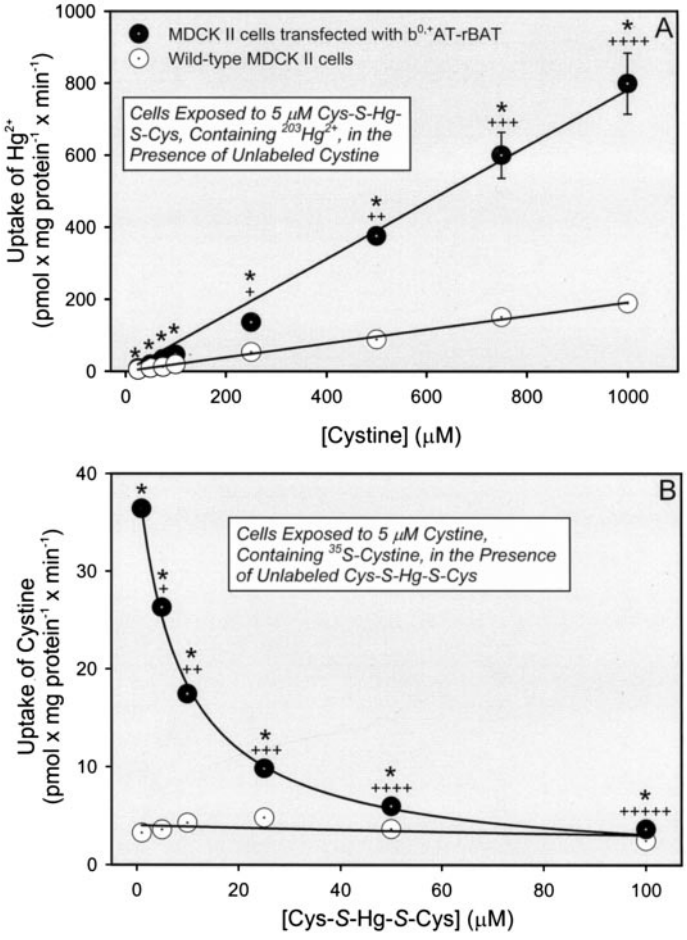

Figure 7. Uptake of 5 μM cystine (containing [^{35}S]cystine) (A) or 5 μM inorganic mercury, as the mercuric conjugate of cysteine, Cys-*S*-Hg-*S*-Cys (containing $^{203}Hg^{2+}$) (B), in the presence of unlabeled Cys-*S*-Hg-*S*-Cys (1 to 100 μM) or unlabeled cystine (25 to 1000 μM), respectively, in wild-type and $b^{0,+}$AT-rBAT-transfected MDCK II cells. Cells were incubated for 30 min at 37°C. Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the same cell type at the initial concentration; ++, significantly different from the mean for the same cell type at each of the preceding concentrations; +++, significantly different from the mean for the same cell type at each of the preceding concentrations; ++++, significantly different from the mean for the same cell type at each of the preceding concentrations; +++++, significantly different from the mean for the same cell type at each of the preceding concentrations.

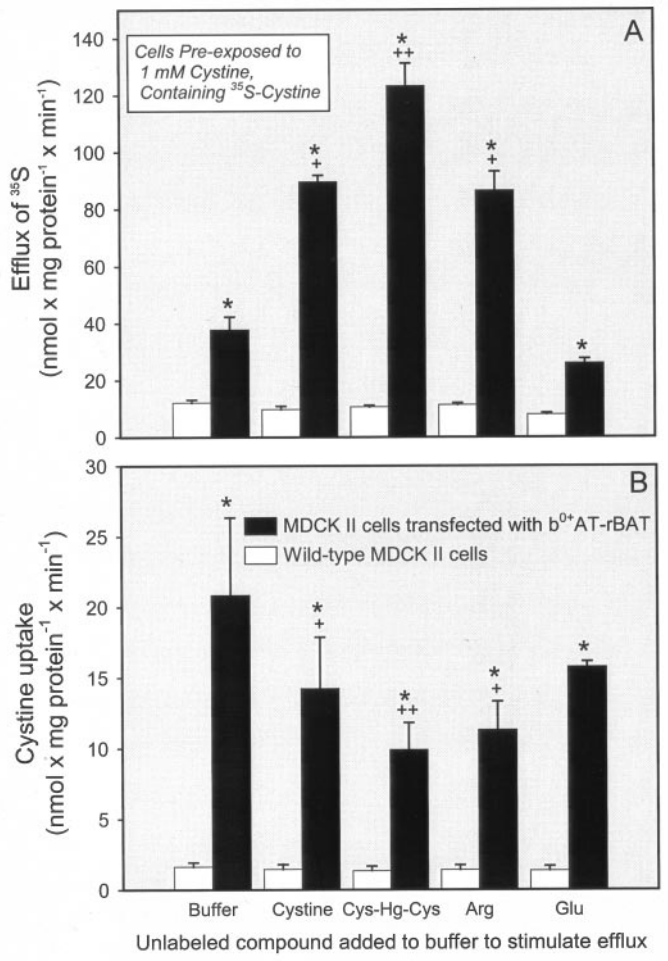

Figure 8. Efflux of ^{35}S (A) and cellular contents of ^{35}S after efflux (B) in wild-type MDCK II cells and $b^{0,+}$AT-rBAT transfectants after exposure to cystine (containing ^{35}S). Cells were exposed to 1 mM cystine for 10 min at 37°C and then incubated with buffer only or 1 mM unlabeled cystine, the mercuric conjugate of cysteine (Cys-*S*-Hg-*S*-Cys), arginine, or glutamate for 1 min at 37°C. Results are presented as means ± SEM. Data represent two experiments performed in duplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the group of transfectants exposed to buffer or glutamate; ++, significantly different from the mean for the transfectants exposed to buffer, cystine, arginine, or glutamate.

$HgCl_2$ and continued to decline with increasing concentrations of $HgCl_2$ (Figure 10C).

Discussion

Because of the growing problem of worldwide contamination of the environment with mercury (23) and the continued use of mercury-containing compounds in industry, medicine, and dentistry, there is an increasing need to better understand how the various chemical forms of this metal are handled by human subjects and other mammals. To truly comprehend how mercury induces its deleterious effects in particular organisms, it is necessary to first understand the mechanisms by which mercuric ions enter the target cells.

In this study, we tested the hypothesis that Cys-*S*-Hg-*S*-Cys is an important, biologically relevant species of Hg^{2+} that acts as a molecular mimic of the amino acid cystine and is transported by the Na^+-independent transporter system $b^{0,+}$. The rationale for studying Cys-*S*-Hg-*S*-Cys rather than other mercuric conjugates is that Cys-*S*-Hg-*S*-Cys is the form of Hg^{2+} most likely presented to the luminal plasma membrane of proximal tubular epithelial cells *in vivo* (2). Furthermore, this study focused on the activity of system $b^{0,+}$ because this

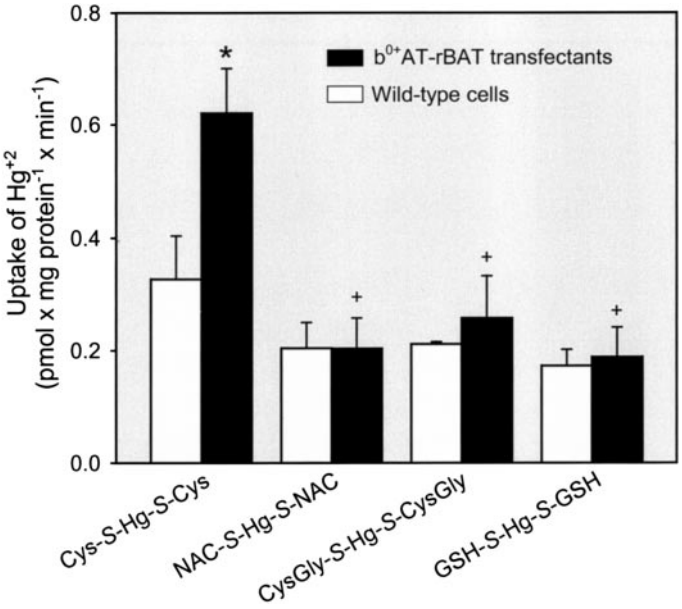

Figure 9. Uptake of inorganic mercury (Hg^{2+}) in wild-type and $b^{0,+}$AT-rBAT-transfected MDCK II cells exposed to 5 μM Hg^{2+} (containing $^{203}Hg^{2+}$) and 20 μM cysteine (Cys), *N*-acetylcysteine (NAC), cysteinylglycine (CysGly), or GSH for 30 min at 37°C. Results are presented as means ± SEM. Data represent three experiments performed in quadruplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the group of transfected cells treated with Cys-*S*-Hg-*S*-Cys.

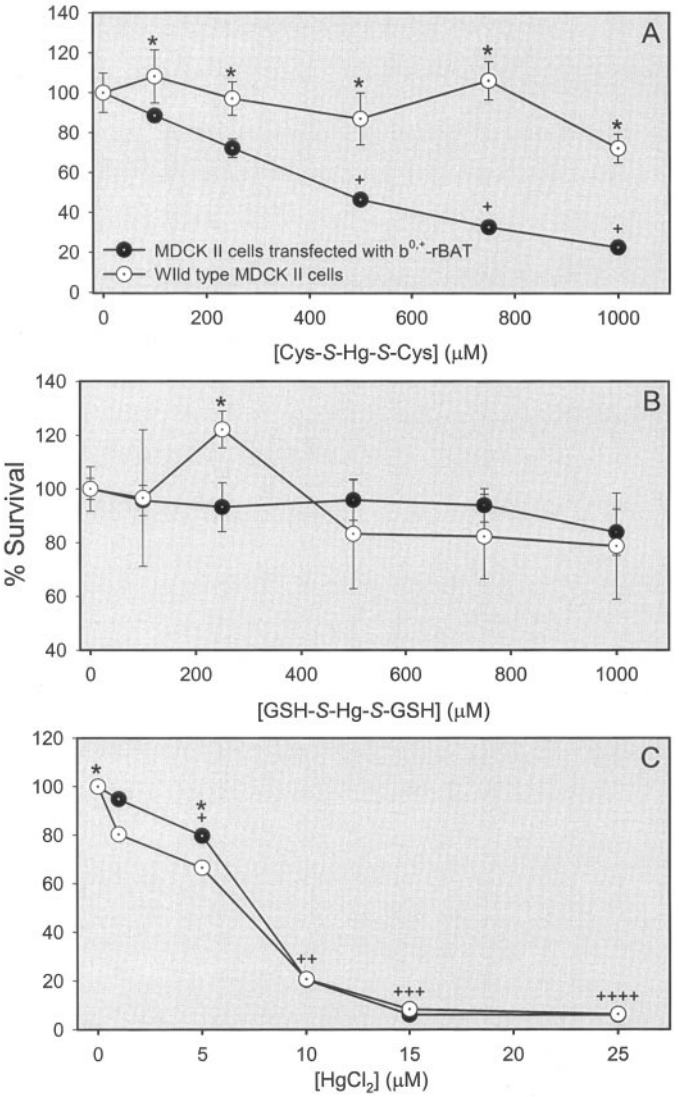

Figure 10. Cellular viability of wild-type cells and $b^{0,+}$AT-rBAT transfectants after 24 h of exposure to various concentrations of Hg^{2+}, in the form of the mercuric conjugate of cysteine (Cys-*S*-Hg-*S*-Cys) (A), the mercuric conjugate of GSH (GSH-*S*-Hg-*S*-GSH) (B), or $HgCl_2$ (C). Results are presented as percent survival. Data represent two experiments performed in duplicate. *, significantly different ($P < 0.05$) from the mean for the corresponding group of wild-type cells; +, significantly different from the mean for the same cell type at the initial concentration; ++, significantly different from the mean for the same cell type at each of the preceding concentrations; +++, significantly different from the mean for the same cell type at each of the preceding concentrations; ++++, significantly different from the mean for the same cell type at each of the preceding concentrations.

transporter is known to transport cystine and is localized to the luminal plasma membrane of the target epithelial cells, *i.e.*, proximal tubular cells (2,13–15).

MDCK II cells, which do not normally express system $b^{0,+}$, were stably transfected with both subunits of this carrier, *i.e.*, $b^{0,+}$AT and rBAT. Laser scanning confocal microscopic analyses demonstrated that $b^{0,+}$AT and rBAT colocalized on the apical plasma membrane of the $b^{0,+}$AT-rBAT transfectants. This distribution reflects the normal localization of these transport proteins in proximal tubular cells *in vivo* (13–15). Cotransfection of MDCK II cells with these two subunits resulted in a gain of function, which was evidenced by a 10-fold increase in the V_{max} for the transport of cystine. Substrate specificity analyses demonstrated that cystine transport in the $b^{0,+}$AT-rBAT transfectants was inhibited by substrates of system $b^{0,+}$, whereas amino acids that are not substrates of system $b^{0,+}$ had no effect on uptake. Analyses of the time and temperature dependence of cystine transport indicated that system $b^{0,+}$ was a functional carrier in the $b^{0,+}$AT-rBAT-transfected cells. Our data confirm that $b^{0,+}$AT-rBAT transfectants represent a reliable *in vitro* model with which to study system $b^{0,+}$-mediated transport. More importantly, these data also provide a foundation for defining the mechanisms involved in the transport of Hg^{2+} in renal epithelial cells.

Various biochemical analyses of the transport of Cys-*S*-Hg-*S*-Cys indicate that this mercuric conjugate is indeed a transportable substrate of system $b^{0,+}$. Time-course analyses in wild-type and $b^{0,+}$AT-rBAT-transfected cells demonstrated that the uptake of Cys-*S*-Hg-*S*-Cys in the transfectants was twofold greater than that in wild-type cells. Analysis of the saturation kinetics of Cys-*S*-Hg-*S*-Cys transport revealed that the V_{max} was approximately twofold greater in the $b^{0,+}$AT-rBAT transfectants than in wild-type cells. In addition, the K_m was approximately one-half lower in the transfectants than in

the corresponding control cells. The lower K_m in the transfectants clearly indicated that another transport system, with a higher affinity for Cys-*S*-Hg-*S*-Cys, was mediating most (if not all) of the uptake of this conjugate in the transfected cells. Because the only apparent difference between the wild-type and transfected MDCK II cells was the presence of a functional system $b^{0,+}$ transporter, it can be concluded that $b^{0,+}$AT-rBAT can mediate the absorptive transport of Cys-*S*-Hg-*S*-Cys. A small amount of Hg^{2+} was associated with wild-type cells exposed to Cys-*S*-Hg-*S*-Cys, which likely represents nonspecific binding and/or uptake via another mechanism.

Analysis of substrate specificity provided substantive support for our hypothesis that Cys-*S*-Hg-*S*-Cys can behave as a functional molecular mimic of cystine at the site of system $b^{0,+}$. Substrates of system $b^{0,+}$ inhibited the transport of both cystine and Cys-*S*-Hg-*S*-Cys, whereas amino acids that are not substrates of this transporter did not affect the uptake of either compound. Therefore, the same carrier (*i.e.*, system $b^{0,+}$) can mediate the uptake of both cystine and Cys-*S*-Hg-*S*-Cys.

Surprisingly, when the uptake of Cys-*S*-Hg-*S*-Cys was assessed in the presence of excess cystine, the inward transport of Cys-*S*-Hg-*S*-Cys was actually stimulated twofold or more in the transfectants. This stimulation was proportional to the concentration of cystine used and was independent of the period of incubation (1 to 30 min; data not shown). As the concentration of unlabeled cystine in the extracellular medium increased, the uptake of Cys-*S*-Hg-*S*-Cys in the transfectants increased. The cystine-induced stimulation of Cys-*S*-Hg-*S*-Cys uptake was specific for this mercuric conjugate. Uptake of other substrates of system $b^{0,+}$, such as [^{35}S]cystine, [^{3}H]arginine, and [^{3}H]lysine, was inhibited when the substrates were presented individually to the transfected cells with unlabeled cystine (data not shown). When the uptake of [^{35}S]cystine was measured in the presence of excess Cys-*S*-Hg-*S*-Cys, transport in the transfectants was inhibited.

These findings indicate that the presence of excess cystine somehow increases the rate of Cys-*S*-Hg-*S*-Cys transport, which is relatively low in the presence of other substrates of system $b^{0,+}$. This enhancement in transport activity could be attributable to efficient exchange of cystine for Cys-*S*-Hg-*S*-Cys at the intracellular binding site of system $b^{0,+}$, which

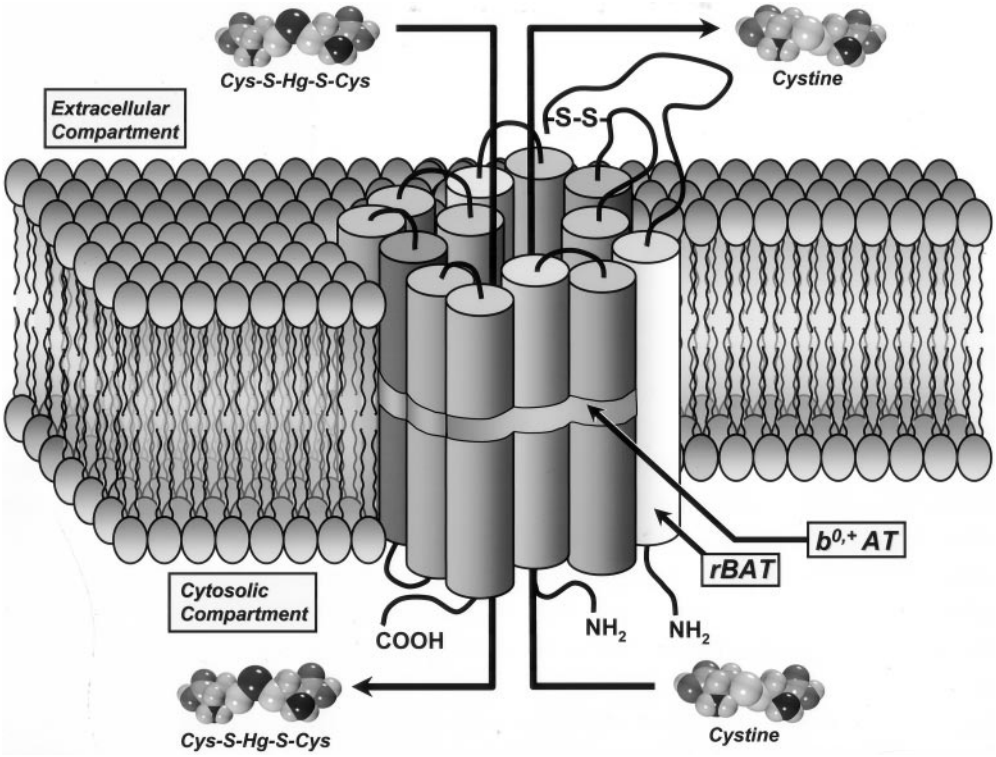

Figure 11. Diagrammatic representation of the transport of cystine and the mercuric conjugate of cysteine (Cys-*S*-Hg-*S*-Cys) via system $b^{0,+}$. The form of Hg^{2+} that is most likely presented to the luminal membrane of proximal tubular epithelial cells *in vivo* is a conjugate of cysteine, primarily in the form of Cys-*S*-Hg-*S*-Cys. As shown in this model, the molecular structure of Cys-*S*-Hg-*S*-Cys is very similar to that of the amino acid cystine (Cys-*S*-*S*-Cys). Given the structural similarity of cystine and Cys-*S*-Hg-*S*-Cys, it is probable that a transport system with a high affinity for cystine mediates the uptake of both compounds. We postulate that a likely candidate for this uptake is system $b^{0,+}$. This carrier is an amino acid exchanger that mediates the transport of cystine, as well as a variety of neutral and cationic amino acids. As a heterodimeric transporter, it is composed of two subunits, $b^{0,+}$AT (shaded cylinders) and rBAT (white cylinder). The linkage of these two subunits, via a disulfide bond (S-S), is essential for the formation of a functional transporter unit. Our data indicate that Cys-*S*-Hg-*S*-Cys is a transportable substrate of system $b^{0,+}$. The data also indicate that Cys-*S*-Hg-*S*-Cys stimulates the efflux of cystine in $b^{0,+}$AT-rBAT-transfected MDCK II cells. Because this efflux is specific to system $b^{0,+}$, it likely that this transporter mediates the exchange of Cys-*S*-Hg-*S*-Cys and cystine.

might promote the intracellular release of Cys-*S*-Hg-*S*-Cys that would otherwise remain associated with the transporter.

To test this theory, the efflux of ^{35}S (to account for potential reduction of cystine to cysteine) was studied in wild-type cells and b^{0+}AT-rBAT transfectants exposed to [^{35}S]cystine. Of the compounds tested, Cys-*S*-Hg-*S*-Cys was the most effective stimulator of ^{35}S efflux. Arginine and cystine stimulated the efflux of ^{35}S from the transfectants but to a lesser degree. Treatment of cells with uptake buffer or glutamate did not significantly stimulate the outward transport of ^{35}S. The intracellular ^{35}S contents corresponded inversely to the pattern of ^{35}S efflux described above, *i.e.*, with greater efflux of ^{35}S, lower cellular ^{35}S contents were observed. Therefore, we conclude that the efflux effect observed in the transfectants is attributable specifically to the activity of system $b^{0,+}$. These data are consistent with the hypothesis that cystine is exchanged intracellularly for Cys-*S*-Hg-*S*-Cys, which facilitates the intracellular release of this conjugate. A diagrammatic representation of this exchange is presented in Figure 11.

Although it is likely that some of the cystine taken up by the MDCK cells was reduced within the cells, it is not clear whether any of the newly formed cysteine could serve as an exchangeable substrate for system $b^{0,+}$. It is unlikely that, if any of the newly formed cysteine was added to the extracellular compartment, this would promote the uptake of cystine, because addition of cysteine to the extracellular compartment was demonstrated to inhibit the uptake of Cys-*S*-Hg-*S*-Cys by system $b^{0,+}$ (Figure 6B). Additional studies are clearly required to further characterize this phenomenon.

Transport of Hg^{2+} in the form of NAC-*S*-Hg-*S*-NAC, Cys-Gly-*S*-Hg-*S*-CysGly, or GSH-*S*-Hg-*S*-GSH was also measured in wild-type cells and transfectants. NAC-*S*-Hg-*S*-NAC is a highly polar homolog of Cys-*S*-Hg-*S*-Cys that is efficiently transported into proximal tubular epithelial cells from the basolateral extracellular compartment by one or more organic anion transporters (2,17). In the lumen, however, the negative charge on each end of the NAC-*S*-Hg-*S*-NAC complex is thought to impede or prevent absorption of this mercuric conjugate across the luminal plasma membrane of renal epithelial cells lining the nephron and collecting ducts. Mercuric conjugates of NAC were used as negative controls in this study, to confirm that the polar nature of NAC-*S*-Hg-*S*-NAC prevents its absorption at the luminal plasma membrane of renal epithelial cells, although NAC-*S*-Hg-*S*-NAC shares structural homology with Cys-*S*-Hg-*S*-Cys. As predicted, when control MDCK II cells or MDCK II cells stably transfected with system $b^{0,+}$ were exposed to NAC-*S*-Hg-*S*-NAC, there was no significant uptake of Hg^{2+} in either cell type.

The rationale for examining the uptake of Hg^{2+} in the form of GSH-*S*-Hg-*S*-GSH or CysGly-*S*-Hg-*S*-CysGly is that these conjugates are putatively present in the proximal tubular lumen *in vivo* and the final degradative product of each of these compounds in the lumen (with the actions of γ-glutamyltransferase and/or cysteinylglycinase) is Cys-*S*-Hg-*S*-Cys (3–6). Because γ-glutamyltransferase and cysteinylglycinase are essentially absent from the luminal compartment of the distal nephron (24) (from which MDCK II cells are derived), GSH-*S*-Hg-*S*-GSH and Cys-Gly-*S*-Hg-*S*-CysGly would not be expected to be enzymatically degraded to Cys-*S*-Hg-*S*-Cys. As predicted, insignificant levels of uptake of Hg^{2+} were detected in either cell type exposed to GSH-*S*-Hg-*S*-GSH or CysGly-*S*-Hg-*S*-CysGly, indicating that neither these conjugates nor NAC-*S*-Hg-*S*-NAC, are transportable substrates of system $b^{0,+}$.

These toxicologic findings indicate that a strong relationship exists between the transport and toxicity of Hg^{2+} as Cys-*S*-Hg-*S*-Cys in the $b^{0,+}$AT-rBAT transfectants. The viability of the transfectants was significantly lower than that of wild-type cells after identical exposures to Cys-*S*-Hg-*S*-Cys. Incubation with GSH-*S*-Hg-*S*-GSH did not significantly alter the cellular viability of either cell type at any concentration studied. This observation was not surprising, since GSH-*S*-Hg-*S*-GSH was not taken up to a significant extent by either cell type. These data indicate that the presence of system $b^{0,+}$ promotes the uptake of Cys-*S*-Hg-*S*-Cys into the intracellular compartment of these cells, which eventually results in cell death.

In conclusion, the results of this study demonstrate for the first time that Cys-*S*-Hg-*S*-Cys is indeed a transportable substrate of system $b^{0,+}$ (Figure 11). Moreover, these results implicate a mechanism of molecular mimicry, with Cys-*S*-Hg-*S*-Cys mimicking the amino acid cystine at the site of system $b^{0,+}$. The transport and toxicologic findings from this study also indicate that system $b^{0,+}$ likely plays a significant role in the nephropathy induced by *in vivo* exposure to Hg^{2+}, by mediating the uptake of a transportable species of Hg^{2+}, Cys-*S*-Hg-*S*-Cys, into proximal tubular epithelial cells.

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