Kidney International, Vol. 68 (2005), pp. 653–664

Cystine and glutamate transport in renal epithelial cells transfected with human system x_c^-

CHRISTY C. BRIDGES and RUDOLFS K. ZALUPS

Division of Basic Medical Sciences, Mercer University School of Medicine, Macon, Georgia

Cystine and glutamate transport in renal epithelial cells transfected with human system x_c^-.

Background. System x_c^- is a heterodimeric transporter, comprised of a light chain, xCT, and heavy chain, 4F2hc, which mediates the sodium-independent exchange of cystine and glutamate at the plasma membrane. In the current study we tested the hypothesis that stable transfection of Madin-Darby canine kidney (MDCK) cells with human xCT and 4F2hc results in the expression of functional system x_c^-.

Methods. MDCK cells were transfected stably with human clones for xCT and 4F2hc. Analyses of time- and temperature-dependence, saturation kinetics, and substrate specificity of L-cystine and L-glutamate transport were carried out in control and xCT-4F2hc-transfected MDCK cells. We also measured the uptake of L-cystine in *Xenopus* oocytes expressing human xCT and/or 4F2hc or xCT and/or rBAT (a heavy chain homologous to 4F2hc).

Results. All of the different sets of data revealed that transport of L-cystine and L-glutamate increased significantly (twofold to threefold) in the MDCK cells subsequent to transfection with xCT-4F2hc. Moreover, uptake of L-cystine also increased (about tenfold) in *Xenopus* oocytes expressing hxCT and h4F2hc. Biochemical analyses of L-cystine uptake in oocytes verified our findings in the transfected MDCK cells. Interestingly, in oocytes injected with rBAT with or without xCT, uptake of L-cystine was significantly greater than that in water-injected oocytes.

Conclusion. Our findings indicate that stable transfection of MDCK cells with xCT and 4F2hc results in a cell-line expressing a functional system x_c^- transporter that can utilize L-cystine and L-glutamate as substrates. This study apparently represents the first stable transfection of a mammalian cell line with system x_c^-.

System x_c^- (designated as such by Makowske and Christensen, [1]) is a sodium (Na^+)-independent cystine/glutamate transporter that has been found in numerous types of mammalian cells [1–6]. This transporter mediates the electroneutral exchange of cystine (which is in a partially deproteinated form having a net charge of –1) and glutamate at the plasma membrane, typically promoting the cellular uptake of cystine through the efflux of glutamate.

Although a number of studies have characterized the transport activity of this system, the molecular identities of the components of system x_c^- were identified only recently. Expression cloning from mouse peritoneal macrophages revealed that there are two distinct proteins that, when expressed together in *Xenopus* oocytes, induced transport activity similar to that described for system x_c^- in native cells [7]. While one of these proteins was identified as the heavy chain of the 4F2 cell surface antigen (4F2hc), the other, designated as xCT (system x_c^- transporter-related protein) was a novel protein [7]. The light chain, xCT, was described as a protein containing 502 amino acids with 12 putative transmembrane domains [7]. Since its initial identification, xCT has been cloned from human fibroblasts [8], human glial cells [9], human retinal pigment epithelium [10], and a human teratocarcinoma cell line, NT2 [11]. As a heterodimeric transporter, system x_c^- requires both, 4F2hc and xCT, for its activity, although xCT appears to be the functional transport unit of this carrier protein.

Under normal physiologic conditions, system x_c^- is expressed ubiquitously at low levels in mammalian cells [12]. However, under conditions of oxidative stress, the transport activity of this carrier appears to be up-regulated [9, 10, 13–20], possibly via an amino acid response element [21]. As a result of this up-regulation, an increased number of cystine molecules could be transported into the cells, which, in turn, would provide (after intracellular reduction) an increased number of molecules of cysteine (the rate-limiting substrate) for glutathione (GSH) synthesis [3, 22]. It should be mentioned that because of the oxidative nature of blood and extracellular compartments, cysteine is supplied primarily to most cells in its oxidized form, cystine.

System x_c^- also plays an important role in the maintenance of other normal cellular homeostatic processes [23, 24]. Given the importance of amino acid transport

Key words: cystine, transport, amino acids, system x_c^-, MDCK cells.

Received for publication January 4, 2005
and in revised form February 22, 2005
Accepted for publication March 22, 2005

and GSH biosynthesis in renal tubular epithelial cells, it is crucial that a thorough and complete understanding of the mechanisms involved in the transport and metabolism of cysteine and cystine be obtained. In renal tubular epithelial cells, a number of cystine transporters have been identified, including systems $b^{0,+}$, $B^{0,+}$, and L [24]. In addition, system x_c^- has been identified in the mouse kidney [7] by Northern blot analyses and in cultured proximal tubular cells (LLC-PK_1) by functional analyses [5].

The role of system x_c^- in the transport of cystine, glutamate, and other amino acids across the plasma membranes of renal epithelial cells has not been well characterized. One of the principal aims of the present study was to determine the mechanistic role of system x_c^- in the transport of L-cystine, L-cysteine and other potential substrates in a line of renal epithelial cells [Madin-Darby canine kidney (MDCK)] transfected stably with both subunits of this transporter. MDCK cells were chosen for this study because they are derived from the distal nephron, and, thus, do not express the same endogenous cystine transporters (e.g., system $b^{0,+}$) that are expressed on the luminal plasma membrane of proximal tubular cells. Moreover, MDCK cells have been transfected successfully with other amino acid transporters and have proven to be reliable models for the study of amino acid transport [25–28]. In the present study, we have successfully created a line of MDCK cells that stably express functional forms of human xCT and human 4F2hc. Using this cell line, we tested the hypothesis that system x_c^- mediates the transport of L-cystine and L-glutamate.

We also utilized oocytes from *Xenopus laevis,* microinjected with the cRNA encoding the subunits of system x_c^-, to study L-cystine and L-glutamate transport. *Xenopus* oocytes represent a nonmammalian model that has been used to study a number of transport systems, including system x_c^- [7, 29]. Since they have very few endogenous transporters, oocytes provide a good model in which to study a selected transporter. In the present study, we used this model as an additional means to test the hypothesis that system x_c^- mediates the transport of L-cystine and L-glutamate. The results of these experiments were also used to validate the results of the experiments carried out in the transfected MDCK cells.

The results from the present study indicate that MDCK cells transfected stably with hxCT and h4F2hc express a functional system x_c^- transporter that mediates Na^+-independent uptake of L-cystine and L-glutamate. Data obtained from experiments in *Xenopus* oocytes support this conclusion. Together, these findings indicate that the MDCK cells transfected stably with xCT-4F2hc are a reliable model in which to study the activity of system x_c^-. To our knowledge, this study represents the first time that human xCT and human 4F2hc have been transfected stably and studied in a mammalian renal epithelial cell line.

METHODS

Tissue culture

MDCK type II cells were kindly provided by Dr. John Pritchard at the National Institute of Environmental Health Sciences (Research Triangle Park, NC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% essential amino acids (Invitrogen, Carlsbad, CA, USA). Cells were passaged by dissociation in 0.25% trypsin (Invitrogen)/0.5 mmol/L ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Stable transfection of MDCK cells

The cDNA transcripts for human xCT (hxCT) and human 4F2hc (h4F2hc) were kindly provided by Dr. Vadivel Ganapathy (Medical College of Georgia, Augusta, GA, USA). The cDNA for hxCT or h4F2hc was subcloned into the *Eco*RI/*Not*I sites of the pcDNA 3.1(+) vector (Invitrogen) that confers resistance to zeocin or geneticin (G418), respectively.

Two days prior to transfection, cells were seeded in 24-well plates at a density of 6×10^4 cells per well. Cells were transfected, according to the manufacturer's instructions, with Lipofectamine 2000 (Invitrogen). Initially, cells were transfected with hxCT, and stable transfectants were selected by culturing cells for 3 weeks in 800 μg/mL zeocin. Following selection, the concentration of zeocin was reduced to 500 μg/mL and the cells were transfected with h4F2hc. Stable transfectants possessing both subunits (xCT-4F2hc transfectants) were selected by culturing cells for 3 weeks in the presence of 500 μg/mL zeocin and 800 μg/mL geneticin. The concentration of geneticin was then reduced to 500 μg/mL. As a negative control, some cells were transfected with each of the pcDNA 3.1(+) vectors (without cDNA inserts) and were selected as described. These cells are hereafter referred to as control cells.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR analyses were carried out to confirm the presence of hxCT and h4F2hc in the xCT-4F2hc transfectants. Total RNA was isolated from the xCT-4F2hc transfectants and control cells using Trizol (Invitrogen) according to the manufacturer's directions. The primers specific for hxCT and h4F2hc have been described previously [10]. As a positive control, the expression of canine glyceraldehyde-3-phosphate dehydrogenase (cGAPDH) was analyzed. The forward primer corresponded to bases

335-356 (5′-GCG GGG CCA AGA GGG TCA TCA T-3′) while the reverse primer corresponded to bases 749-727 (5′-GCT TTC TCC AGG CGG CAG GTC AG-3′).

Evaluation of transport

In the current study, we analyzed the uptake of radiolabeled L-cystine and L-glutamate in order to thoroughly characterize the activity of system x_c^- in control cells and xCT-4F2hc transfectants. Since system x_c^- is an exchanger of cystine and glutamate, the activity of this carrier can be monitored by measuring the uptake of radiolabeled L-cystine or L-glutamate. Indeed, previous studies in retinal pigment epithelial cells have utilized radiolabeled glutamate to measure successfully the activity of system x_c^- [10, 14]. It should be noted that, for the purpose of the current study, we assumed that extracellular L-cystine or L-glutamate could exchange for intracellular L-glutamate or L-cystine, respectively.

Uptake measurements were performed as described previously [10, 14, 26, 27]. Briefly, culture media was aspirated from wells and cells were washed with warm uptake buffer lacking Na^+ [25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (Hepes)/Tris, 140 mmol/L *N*-methyl-D-glucamine chloride, 5.4 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 0.8 mmol/L $MgSO_4$, and 5 mmol/L glucose, pH 7.5]. Uptake was initiated by adding 250 μL of uptake buffer containing [^{35}S]-L-cystine (0.6 mCi/mg; 0.125 μCi/well) or [^{3}H]-L-glutamic acid (33.3 mCi/mg; 0.0625 μCi/well) (Amersham Biosciences, Piscataway, NJ, USA). After incubation for the desired time, the radiolabeled compounds were removed and the cells were washed twice with ice-cold uptake buffer. Cells were then solubilized with 1% sodium dodecyl sulfate (SDS) in 0.2 N NaOH and the cellular lysate was added to 5 mL of Opti-Fluor scintillation cocktail (Perkin Elmer, Shelton, CT, USA). The radioactivity contained therein was determined by counting samples in a Beckman LS6500 scintillation counter (Beckman Instruments, Fullerton, CA, USA).

Microinjection of *Xenopus laevis* oocytes

Ovaries were isolated from sexually mature, female *Xenopus laevis* (Xenopus One, Ann Arbor, MI, USA) and defolliculated as described previously [30]. Briefly, this process employs digestion of connective tissue with 1 mg/mL collagenase A (Roche Molecular Biochemicals, Indianapolis, IN, USA) followed by incubation in 100 mmol/L K_2HPO_4. Following isolation, the oocytes were placed in Oocyte Ringer's 2 (OR-2) (82.5 mmol/L NaCl, 2.5 mmol/L KCl, 1 mmol/L Na_2HPO_4, 3 mmol/L NaOH, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 1 mmol/L Na-pyruvate, and 5 mmol/L Hepes, pH 7.6) supplemented with 5% horse serum (Atlanta Biologicals) and 50 μg/mL gentamycin sulfate (Invitrogen) and stored overnight in a shaking incubator at 18°C. Stage IV and V oocytes were then microinjected with 23 nL of either molecular biology grade water or capped RNA encoding hxCT, h4F2hc, or hrBAT at a concentration of 1.24 μg/μL.

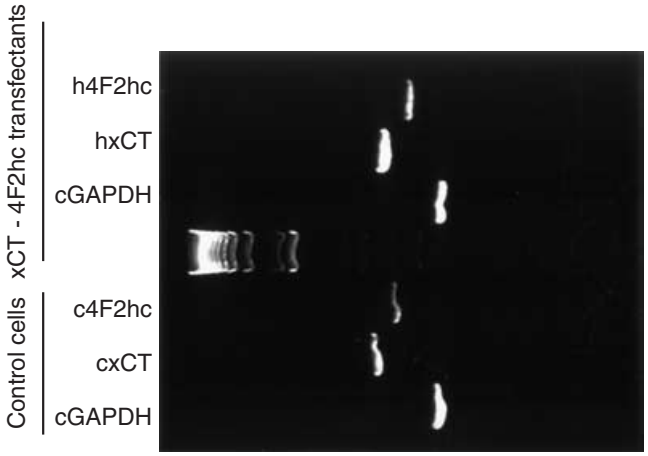

Fig. 1. Photographic presentation of reverse transcription-polymerase chain reaction (RT-PCR) analyses for human xCT(hxCT) and canine xCT (cxCT), human 4F2hc (h4F2hc) and canine 4F2hc (c4F2hc), and canine glyceraldehyde-3-phosphate dehydrogenase (cGAPDH) in control and xCT-4F2hc–transfected Madin-Darby canine kidney (MDCK) cells. The expected sizes of the RT-PCR products, as predicted from the positions of the primers, were 724 bp for hxCT, 603 bp for h4F2hc, and 415 bp for cGAPDH.

Transport experiments with the oocytes were conducted three days following injection. Prior to use, the oocytes were separated into groups of ten in 24-well plates and were washed with 1 mL of uptake buffer. Then, 350 μL of uptake buffer containing 5 μmol/L L-cystine (containing [^{35}S]-L-cystine) were added to each well and incubated for 30 min at room temperature. Following incubation, oocytes were rinsed twice with 1 mL of ice-cold uptake buffer. Oocytes were then lysed in 0.5 mL of 1% SDS/0.2 N NaOH. After complete lysis, the radioactivity of the samples was determined by liquid scintillation spectroscopy as described above.

Data analyses

All experiments were performed in triplicate or quadruplicate and were repeated at least twice for confirmation. Data for each parameter assessed ($N = 3$ or 4) were first analyzed using a two-way or three-way analysis of variance (ANOVA). Tukey's multiple comparison procedure was used to assess differences among the means. A P value of < 0.05 was considered statistically significant.

RESULTS

RT-PCR

The presence of mRNA transcripts encoding hxCT and h4F2hc was confirmed using RT-PCR analyses (Fig. 1). A 724 bp fragment was obtained for hxCT while a 603 bp

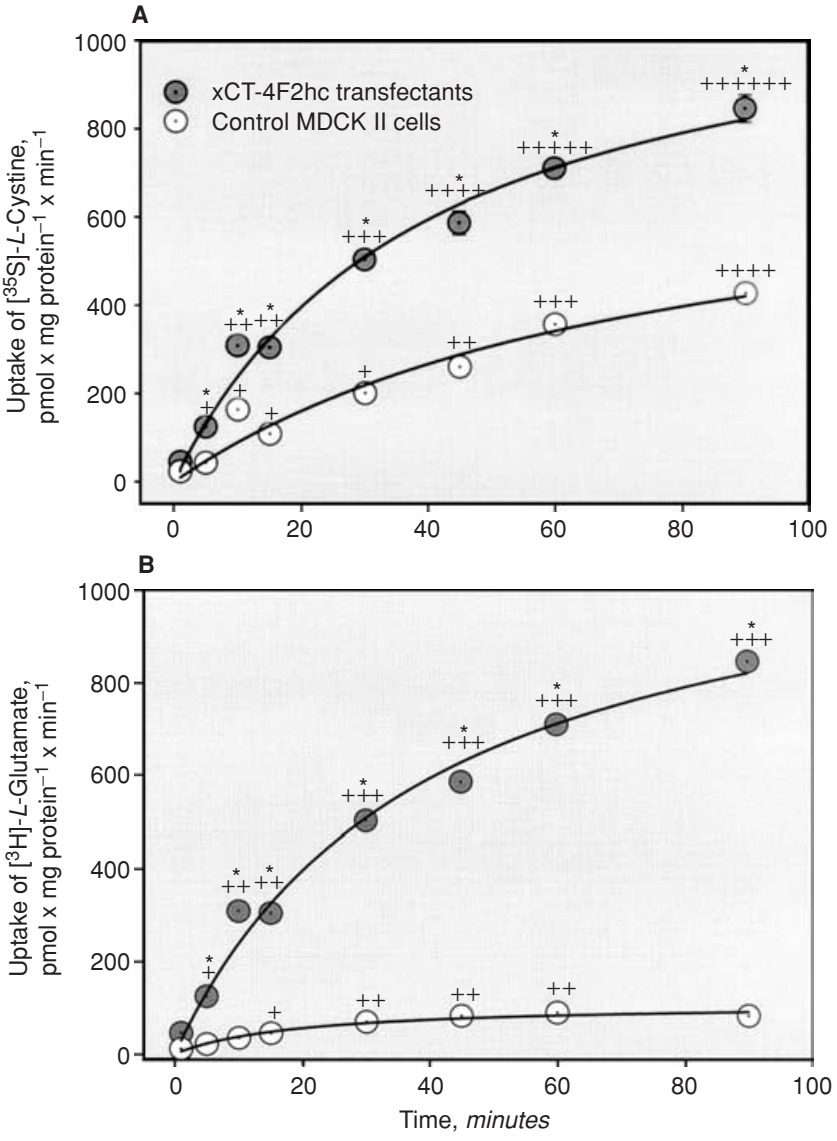

Fig. 2. Uptake of [^{35}S]-L-cystine or [^{3}H]-L-glutamate in control and xCT-4F2hc–transfected Madin-Darby canine kidney (MDCK) cells. Cells were exposed to 5 μmol/L [^{35}S]-L-cystine (*A*) or [^{3}H]-L-glutamate (*B*) at 37°C for time periods ranging from 5 to 90 minutes. Samples were taken for determinations at indicated times. Data are presented as mean ± SE for four samples. Each experiment was performed at least twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control cells; + Significantly different from the mean for the same cell type at the initial time point; ++, +++, ++++, +++++, or ++++++ Significantly different from the mean for the same cell type at each of the preceding time points.

fragment was obtained for h4F2hc. Transcripts encoding these proteins were identified in both the control cells and the xCT-4F2hc transfectants; however, the intensity of the bands was greater in the transfectants. A 415 bp fragment was observed for cGAPDH. There was no detectable difference in the expression of cGAPDH between the control cells and the xCT-4F2hc transfectants.

Characterization of system x_c^- activity in MDCK transfectants

Time course analyses were carried out in control and xCT-4F2hc–transfected MDCK cells. The uptake of [^{35}S]-L-cystine (Fig. 2A) or [^{3}H]-L-glutamate (Fig. 2B) increased in both types of cells over the course of 90 minutes. However, the uptake of each compound was significantly greater in the xCT-4F2hc transfectants than in the control cells at nearly every time examined.

The Michaelis-Menten kinetics of L-cystine (Fig. 3A) or L-glutamate (Fig. 3B) transport was also measured in control and xCT-4F2hc–transfected cells. As with the time course results, the uptake of [^{35}S]-L-cystine or [^{3}H]-L-glutamate was significantly greater in the transfectants than in the control cells at almost every concentration studied. The estimated maximum velocity (V_{max}) of [^{35}S]-L-cystine transport was 589.9 ± 26 pmol × mg protein^{-1} × min^{-1} in the xCT-4F2hc transfectants and 322.5 ± 30.1 pmol × mg protein^{-1} × min^{-1} in the control cells. The Michaelis-Menten constant (K_m) was calculated to be 121.0 ± 17.8 μmol/L for the xCT-4F2hc transfectants and 164.2 ± 47.5 μmol/L for the control cells. Regarding the uptake of [^{3}H]-L-glutamate, the V_{max} was 2.06 ± 0.18 nmol × mg protein^{-1} × min^{-1} in the

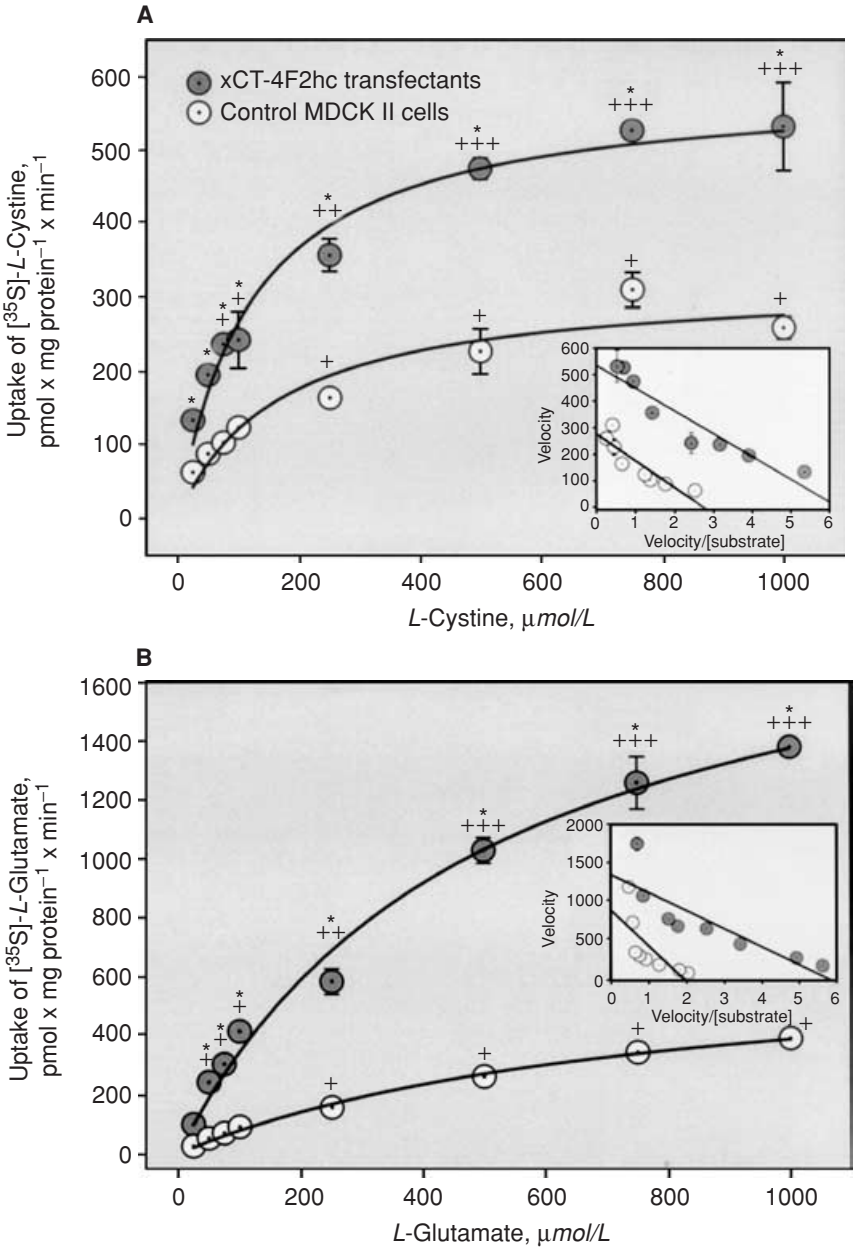

Fig. 3. Saturation kinetics for the transport of [^{35}S]-L-cystine or [^{3}H]-L-glutamate in control and xCT-4F2hc–transfected Madin-Darby canine kidney (MDCK) cells. Cells were incubated for 15 minutes at 37°C with 5 μmol/L [^{35}S]-L-cystine (*A*) or [^{3}H]-L-glutamate (*B*) in the presence of unlabeled L-cystine or L-glutamate, respectively. Each inset represents an Eadie-Hofstee plot of the data. Results are presented as mean ± SE for four samples. Each experiment was performed twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control cells; + Significantly different from the mean for the same cell type at the initial concentration; ++ or +++ Significantly different from the mean for the same cell type at each of the preceding concentrations.

xCT-4F2hc transfectants and 0.68 ± 0.05 nmol × mg protein^{-1} × min^{-1} in the control cells. The K_m was estimated to be 0.50 ± 0.09 mmol/L in the transfectants and 0.76 ± 0.11 mmol/L in the control cells.

The temperature dependence of the activity of system x_c^- was measured by analyzing the saturation kinetics for the uptake of [^{35}S]-L-cystine (Fig. 4A) or [^{3}H]-L-glutamate (Fig. 4B) in control MDCK cells and xCT-4F2hc transfectants at 4°C and 37°C. The uptake of either substrate at 37°C was significantly greater in the transfectants than in the corresponding group of control cells at every concentration studied. When the experimental temperature was maintained at 4°C, transport activity was negligible in both cell types. Additionally, there were no significant differences in the accumulation of cystine or glutamate between corresponding groups of transfected and control cells.

Figure 5 shows substrate-specificity analyses for the transport of L-cystine (Fig. 5A) or L-glutamate (Fig. 5B), in the absence of Na^+, in control cells and xCT-4F2hc transfectants. The uptake of [^{35}S]-L-cystine in the transfectants was reduced significantly by the presence of unlabeled L-cysteine, L-cystine, DL-homocysteine, L-glutamate, L-serine, L-leucine, L-phenylalanine, L-proline, L-alanine, and L-aspartate. With the exception of cells exposed to L-cystine, L-cysteine, L-glutamate, or L-lysine, there were no significant differences in the uptake of L-cystine among the groups of control

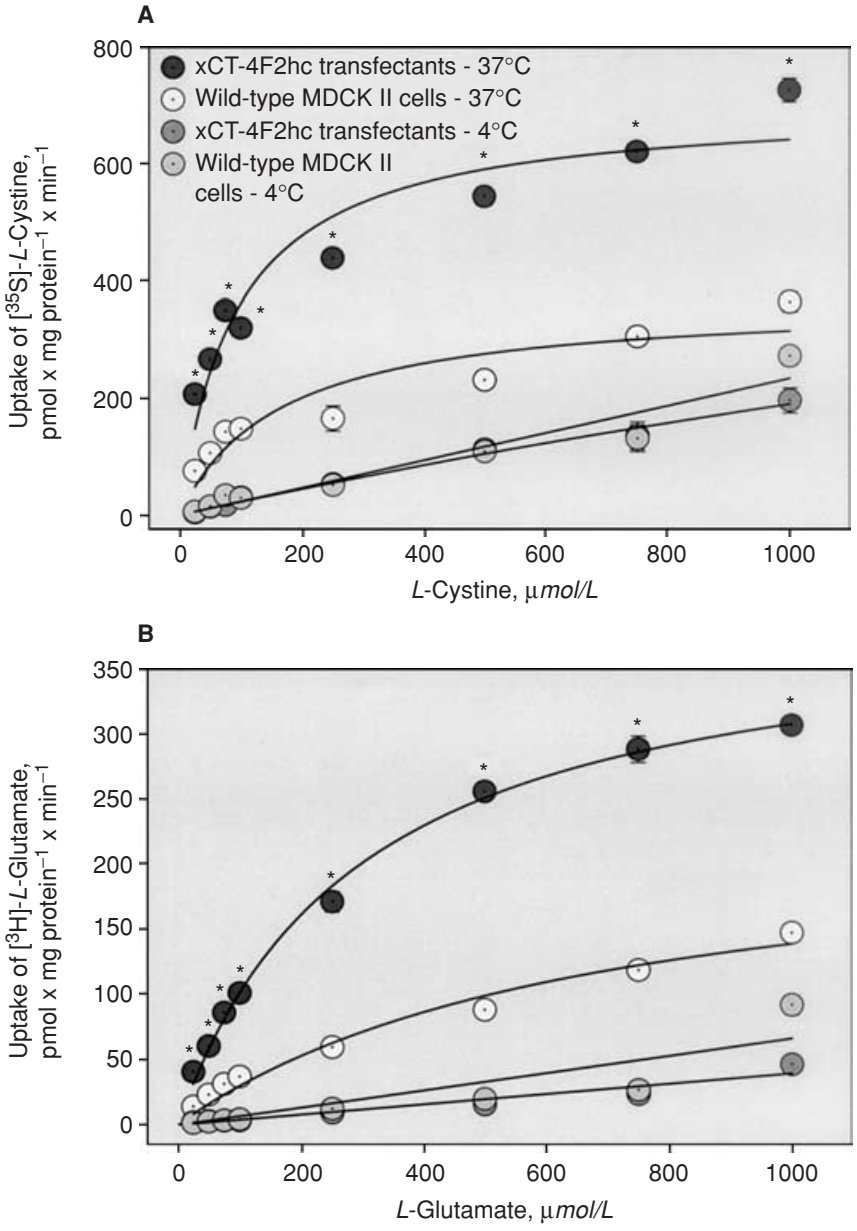

Fig. 4. Effect of temperature on the uptake of cystine or glutamate in control and xCT-4F2hc–transfected Madin-Darby canine kidney (MDCK) cells. Cells were exposed to 5 μmol/L [^{35}S]-L-cystine (*A*) or [^{3}H]-L-glutamate (*B*) for 15 minutes at 37°C or 4°C in the presence of unlabeled L-cystine or L-glutamate, respectively. Data are presented as mean ± SE for four samples. Each experiment was performed at least twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the corresponding group of control cells.

cells. The pattern of amino acid inhibition of [^{3}H]-L-glutamate uptake was similar to that of L-cystine. The transport of glutamate was reduced significantly by L-cysteine, L-cystine, L-glutamate, L-serine, L-leucine, L-phenylalanine, L-proline, L-alanine, and L-aspartate. In the corresponding control cells, only L-cysteine, L-cystine, and L-glutamate inhibited significantly the uptake of L-glutamate.

Characterization of system x_c^- activity in *Xenopus laevis* oocytes

Time course analyses were carried out in *Xenopus* oocytes injected simultaneously with RNA encoding hxCT and h4F2hc. A fifteen-fold increase in the uptake of [^{35}S]-L-cystine (Fig. 6) was observed in the oocytes injected with hxCT and h4F2hc relative to that in water-injected controls.

In addition, analyses of the saturation kinetics for the uptake of L-cystine (Fig. 7) were carried out in water-injected and hxCT-h4F2hc–injected oocytes. At every concentration examined, the uptake of [^{35}S]-L-cystine was significantly greater in the hxCT-h4F2hc–injected oocytes than in the water-injected oocytes. The V_{max} for the uptake in the hxCT-h4F2hc–injected oocytes was calculated to be 293.2 ± 39.3 pmol × mg protein^{-1} × min^{-1} while the K_m was estimated to be 120.9 ± 51.8 μmol/L.

Sodium-independent, substrate-specificity analyses of L-cystine uptake (Fig. 8) were also carried out in

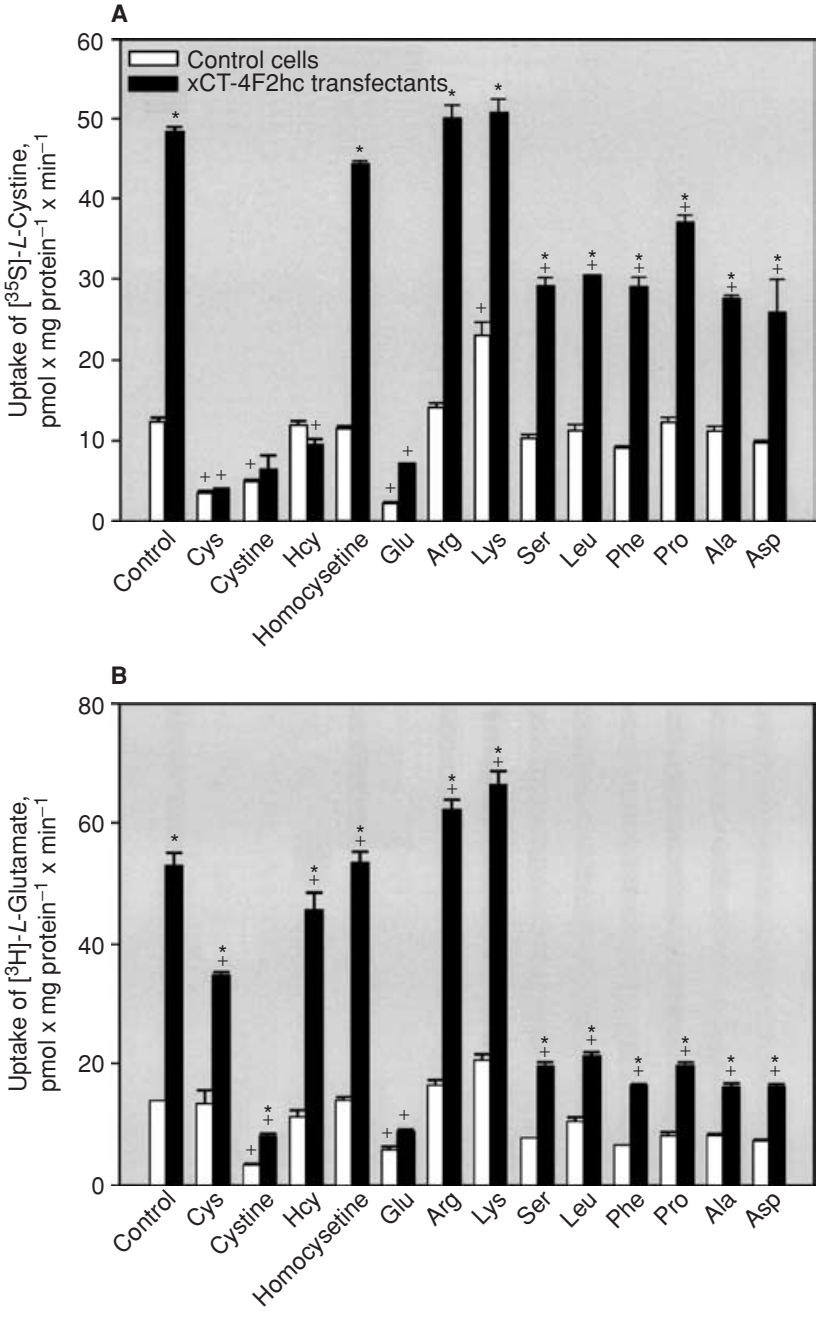

Fig. 5. Substrate specificity analyses of the sodium-independent transport of [^{35}S]-L-cystine or [^{3}H]-L-glutamate in control and xCT-4F2hc–transfected Madin-Darby canine kidney cells. Cells were incubated for 15 minutes at 37°C with 5 µmol/L [^{35}S]-L-cystine (*A*) or [^{3}H]-L-glutamate (*B*) in the presence of various unlabeled amino acids (3 mmol/L) (unlabeled L-cystine and DL-homocystine, 1 mmol/L). Data are presented as mean ± SE for four samples. Each experiment was performed at least twice for the purpose of validation. ∗Significantly different ($P < 0.05$) from the mean for the corresponding group of control cells; +Significantly different from the mean for the control group of the corresponding cell type.

water- and hxCT-h4F2hc–injected oocytes. These analyses demonstrated that the amino acids, L-cysteine, L-cystine, DL-homocysteine, L-glutamate, L-leucine, L-alanine, and L-aspartate were able to inhibit significantly the uptake of [^{35}S]-L-cystine in the hxCT-h4F2hc–injected oocytes, while there was no significant inhibition in corresponding water-injected controls.

As a negative control, some oocytes were injected with hxCT or h4F2hc alone (Fig. 9A). The uptake of [^{35}S]-L-cystine in these groups of oocytes was not significantly different from that in corresponding groups of oocytes injected with water. Under certain conditions (possibly pathologic), the heavy chain, rBAT, may associate with a light chain subunit other than its normal counterpart, $b^{0,+}$AT. To test this possibility, the uptake of [^{35}S]-L-cystine was measured in oocytes injected simultaneously with hxCT and hrBAT or with hrBAT alone (Fig. 9A). Simultaneous injection of hxCT and hrBAT resulted in a ninefold increase in the uptake of cystine compared with that in water-injected controls. Interestingly, the

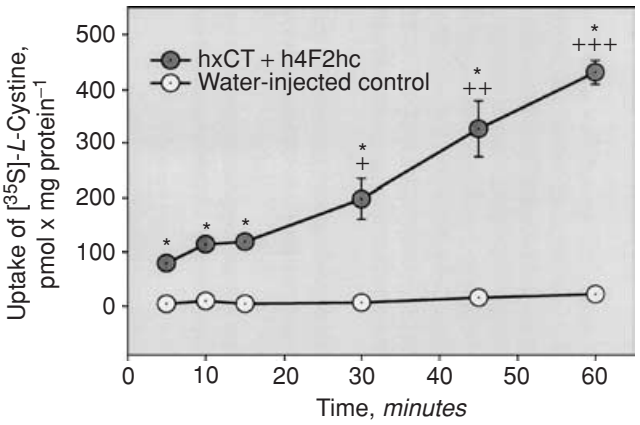

Fig. 6. Uptake of cystine in *Xenopus* oocytes injected simultaneously with human xCT (hxCT) and human 4F2hc (h4F2hc) or water. Oocytes were exposed to 5 μmol/L [^{35}S]-L-cystine at room temperature for time periods ranging from 5 to 60 minutes. Samples were taken for estimation at indicated time points. Data are presented as mean ± SE of three samples, with each sample obtained from 10 oocytes per well. Each experiment was also performed at least twice for the purpose of validation. ∗Significantly different ($P < 0.05$) from the mean for the corresponding group of water-injected oocytes; +Significantly different from the mean for the same set of oocytes at the initial time point; ++ or +++Significantly different from the mean for the same set of oocytes at each of the preceding time points.

uptake of [^{35}S]-L-cystine in oocytes expressing hrBAT only was approximately sevenfold greater than that of water-injected controls.

In order to determine the possible transport systems that mediate the uptake of L-cystine in oocytes injected with either xCT and 4F2hc, xCT and rBAT, rBAT alone, or water, the transport of [^{35}S]-L-cystine was measured in the presence of L-glutamate or L-arginine (Fig. 9B). L-glutamate, which is a characteristic substrate of system x_c^- inhibited significantly the uptake of [^{35}S]-L-cystine in the oocytes injected with xCT and 4F2hc, but did not inhibit this uptake in other groups of oocytes. Alternatively, L-arginine, which is a substrate of system $b^{0,+}$, inhibited significantly the uptake of [^{35}S]-L-cystine in oocytes injected with xCT and rBAT or rBAT alone, but had no effect on this uptake in oocytes injected with xCT and 4F2hc.

DISCUSSION

Cystine is an amino acid essential for the maintenance of numerous vital cellular activities. As a result, various organs in the body attempt to conserve this amino acid by means of absorptive transport. In the kidneys, the cystine that is ultra-filtered at the glomeruli is reclaimed by absorptive transport primarily along the pars convoluta and pars recta of the proximal tubule. Genetic defects affecting the luminal absorption of cystine in the proximal tubules of humans is of clinical importance, inasmuch as cystinuria is induced, which increases the risk of intrarenal development of cystine calculi [31]. Accordingly, the primary focus of most studies on renal cystine transport has been on the luminal handling of this amino acid. Not surprisingly, though, cystine is also taken up from the peritubular capillaries at the basolateral plasma membrane. This probably occurs in all of the epithelial cells lining the nephron and collecting duct, since this form of uptake likely represents the means by which these epithelial cells can obtain cystine for nutritive and homeostatic purposes [24].

One potential mechanism involved in the basolateral transport of cystine is the cystine/glutamate transporter, system x_c^-. This transporter has been studied in various types of cells, yet little is known about its activity in the epithelial cells lining the various segments of the nephron. Therefore, one of the aims of the present study was to characterize the activity of this carrier in a line of renal epithelial cells. MDCK cells were chosen for this study because they are derived from the distal nephron, where there is little, to no, absorptive (luminal) transport of cystine. We tested the hypothesis that cystine and glutamate are high affinity substrates of system x_c^- when this transporter is expressed in MDCK cells transfected stably with both subunits of this carrier. This study is novel in that we have characterized the uptake of L-cystine and L-glutamate in an epithelial cell-line that has been genetically manipulated to overexpress system x_c^-. It is important to note that many of the previous studies of this carrier were carried out in cell lines that expressed this, and other, cystine transporter(s) natively. In the present study, we utilized a renal epithelial cell line derived from the distal nephron (MDCK cells). These epithelial cells express relatively fewer native cystine transporters than the epithelial cells lining the proximal tubule. Stable transfection of these cells with system x_c^- provides a means to study the activity of this carrier by measuring differences in L-cystine transport between transfectants and control cells. To our knowledge, we are the first to characterize the uptake of L-cystine and L-glutamate in a mammalian epithelial cell line transfected stably with system x_c^-.

RT-PCR analyses of xCT and 4F2hc in the control and xCT-4F2hc–transfected MDCK cells indicate that system x_c^- is overexpressed in the xCT-4F2hc transfectants. Although transcripts encoding xCT and 4F2hc were identified in the control cells, the overexpression resulting from the stable transfection of xCT and 4F2hc allowed us to study selectively the activity of system x_c^- in this line of cells. Since the only apparent difference between the xCT-4F2hc transfectants and the control cells is the presence of additional transcripts encoding xCT and 4F2hc, differences in the transport of L-cystine and L-glutamate can be attributed to the activity of system x_c^-. Furthermore, overexpression of system x_c^- in MDCK cells results in the probable localization of this carrier in both luminal

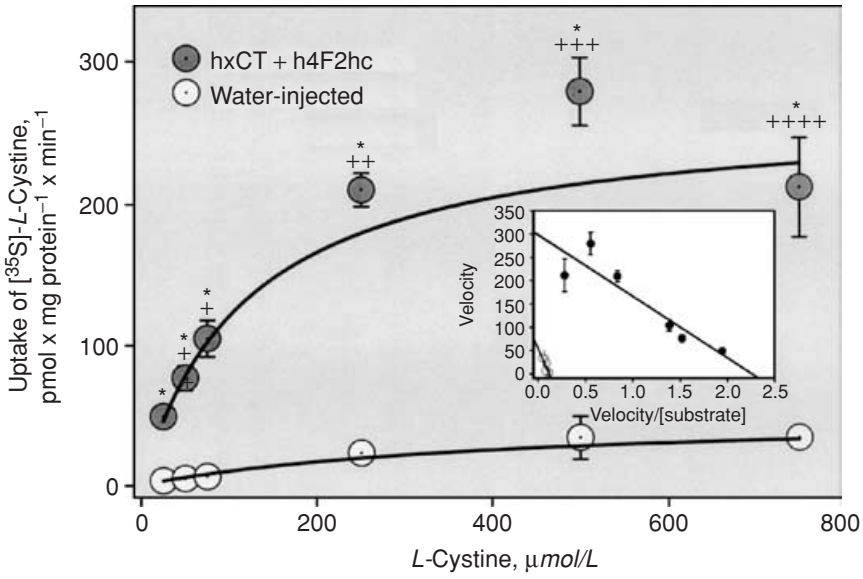

Fig. 7. Saturation kinetics of cystine uptake in *Xenopus* oocytes injected simultaneously with human xCT (hxCT) and human 4F2hc (h4F2hc) or water. Oocytes were exposed to 5 μmol/L [^{35}S]-L-cystine for 30 minutes at room temperature in the presence of unlabeled cystine (inset, Eadie-Hofstee plot). Data are presented as mean ± SE of three samples, with each sample obtained from 10 oocytes per well. Each experiment was also performed at least twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the corresponding group of water-injected oocytes; + Significantly different from the mean for the same set of oocytes at the initial concentration; ++ or +++ Significantly different from the mean for the same set of oocytes at each of the preceding concentrations.

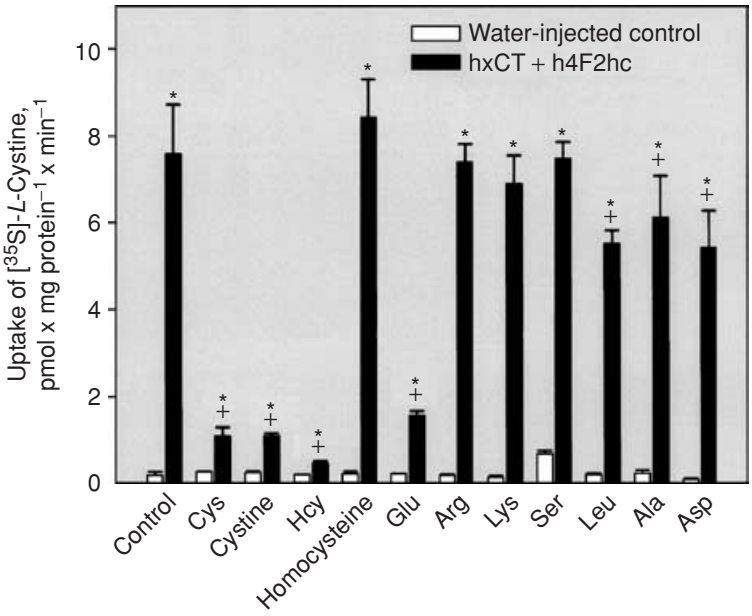

Fig. 8. Analyses of sodium-independent, substrate specificity of L-cystine uptake in *Xenopus* oocytes injected simultaneously with human xCT (hxCT) and human 4F2hc (h4F2hc) or water. Oocytes were exposed to 5 μmol/L [^{35}S]-L-cystine for 30 minutes at room temperature in the presence of unlabeled amino acids (3 mmol/L) (L-cystine, DL-homocystine, 1 mmol/L). Data are presented as mean ± SE of three samples, with each sample obtained from 10 oocytes per well. Each experiment was also performed at least twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the corresponding group of water-injected oocytes; + Significantly different from the mean for the control group of corresponding oocytes.

and basolateral plasma membranes, and thus, allows us access to a transporter that is localized normally to the basolateral plasma membrane of most epithelial cells.

Time course analyses revealed that the rate of uptake of L-cystine and L-glutamate was at least twofold greater in the xCT-4F2hc transfectants than in control cells. Analyses of the saturation kinetics for the transport of cystine also showed a twofold or greater increase in uptake in the transfectants compared with that in control cells. Experiments measuring the uptake of L-cystine in *Xenopus* oocytes validated these findings. The apparent K_m (a measure of substrate affinity) for L-cystine transport in the transfectants (121.0 ± 17.8 μmol/L) (Fig. 2) was nearly identical to that calculated for the uptake of L-cystine in *Xenopus* oocytes (120.9 ± 51.8 μmol/L) (Fig. 8). Moreover, these values were similar to those obtained in other types of nontransfected cells [5, 32].

It is important to note that the apparent K_m for the transport of L-cystine was lower in the xCT-4F2hc transfectants (121.0 ± 17.8 μmol/L) than in control cells (164.2 ± 47.5 μmol/L). This was also true of the K_m for L-glutamate transport (0.76 ± 0.11 mmol/L for control cells and 0.50 ± 0.09 mmol/L for the transfectants). These findings were somewhat surprising since MDCK cells express system x_c^- endogenously. We originally anticipated that the K_m would be similar in both types of cells since the same transport system is ostensibly involved in the uptake of L-cystine. A possible explanation for this shift in affinity is that, in control cells, a larger fraction of L-cystine uptake is mediated by endogenous transporters, other than

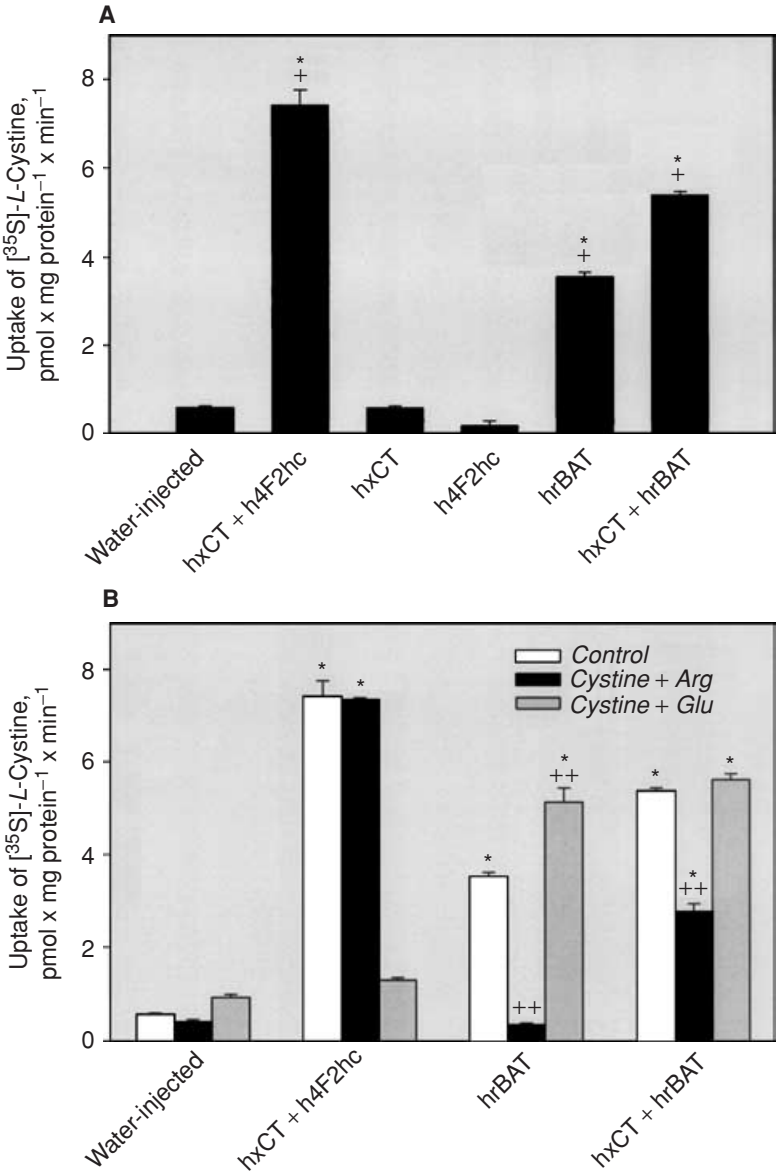

Fig. 9. Uptake of L-cystine in *Xenopus* oocytes injected with various cDNA constructs. (*A*) Oocytes were injected with either water, human xCT (hxCT), human 4F2hc (h4F2hc), human rBAT (hrBAT) or simultaneously with hxCT and h4F2hc or hxCT and hrBAT and exposed to 5 μmol/L [^{35}S]-L-cystine for 30 minutes at room temperature. (*B*) Oocytes were injected with either water, hxCT, and h4F2hc, or hxCT and hrBAT and were exposed to 5 μmol/L [^{35}S]-L-cystine for 30 minutes at room temperature in the presence of 3 mmol/L unlabeled arginine or glutamate. Data are presented as mean ± SE of three samples, with each sample obtained from 10 oocytes per well. Each experiment was also performed at least twice for the purpose of validation. *Significantly different ($P < 0.05$) from the mean for the water-injected oocytes; + Significantly different from all other groups of oocytes; ++ Significantly different from the mean for the control oocytes injected with the same RNA transcripts.

system x_c^-. The affinity of these transporters for cystine would likely be lower than that of system x_c^-. Transfection of xCT and 4F2hc may have permitted a greater percentage of the total pool of transported L-cystine to be taken up by system x_c^- and less by endogenous transporters. This would have resulted in a lower K_m (i.e., higher affinity) for the transport of L-cystine in the transfectants.

In addition to the aforementioned time course and kinetic analyses, data from substrate specificity experiments provide substantive evidence supporting the reliability of the xCT-4F2hc transfectants as models in which to study the activity of system x_c^-. Compounds (e.g., L-cystine, L-glutamate, L-aspartate, L-serine, L-alanine) that have been identified as substrates of system x_c^- [2–4, 6, 8–10, 32] inhibited the transport of L-cystine and L-glutamate; whereas compounds that are not substrates of this carrier did not affect the uptake of these amino acids. This pattern of amino acid inhibition was also observed in experiments utilizing *Xenopus* oocytes, which validates the transport data in the MDCK cells. In addition, the pattern of amino acid inhibition of L-cystine transport remained the same in the presence and absence of Na^+ ions, despite the fact that transport activity was greater in the presence of Na^+ (data not shown). The increased rate of uptake in the presence of Na^+ is likely due to the activities of Na^+-dependent amino acid transporters, such as systems A, or ASC, all of which have been identified in MDCK cells [33].

Data from the current study also indicate that both xCT and 4F2hc are required for system x_c^- to function properly. Indeed, our data show that the uptake of L-cystine or L-glutamate was greater in *Xenopus* oocytes injected

simultaneously with xCT and 4F2hc than in oocytes injected with either xCT or 4F2hc alone. Preliminary experiments in MDCK cells transfected with either xCT or 4F2hc suggest a similar trend.

Moreover, this study examined the ability of xCT to function with rBAT, a heavy chain subunit localized exclusively to the luminal plasma membrane of proximal tubular cells. This subunit, also named NBAT or D2, interacts normally with $b^{0,+}$AT to form system $b^{0,+}$ and possesses approximately 25% sequence identity with 4F2hc [34]. Because of their homology and functional similarities, these two subunits may be interchangeable with xCT under certain conditions. Indeed, a previous in vitro study using transiently transfected human retinal pigment epithelial cells and microinjected *Xenopus* oocytes, showed that a $b^{0,+}$AT-like protein is capable of functioning with 4F2hc, rather than rBAT [35]. Given this finding, it is plausible to suggest that rBAT may interact with light chains that normally associate with 4F2hc. Indeed, transient transfection of cultured NIH3T3 cells and microinjection of *Xenopus* oocytes with rBAT and xCT induced transport activity like that of system x_c^- [36]. Similarly, our data show that simultaneous injection of xCT and rBAT into *Xenopus* oocytes, results in the ability of these cells to take up L-cystine. Interestingly, unlike the aforementioned study, our data show that the uptake of L-cystine by oocytes injected with xCT and rBAT was not inhibited by L-glutamate, which is a competitive inhibitor of system x_c^-. Thus, this uptake does not appear to be mediated by system x_c^-. Alternatively, L-arginine, a competitive inhibitor of system $b^{0,+}$, blocked the uptake of *L*-cystine by 50%. Therefore, it appears that at least half of this uptake is due to the activity of system $b^{0,+}$, while the transport system(s) responsible for the remaining fraction is/are unknown. It should be pointed out that an association between xCT, which is normally a basolateral protein, and rBAT, which is localized in the luminal membrane, has not been demonstrated in vivo. Yet, given the current data, we can hypothesize that, within proximal tubular cells, xCT may associate with rBAT, which may consequently alter the membrane distribution of either protein. As these two proteins distribute normally on opposite plasma membranes, they may associate under pathological conditions that result in a loss of cellular polarity. Our findings, therefore, may aid in explaining some of the processes that occur when cells undergo pathologic changes.

It is interesting to note that injection of *Xenopus* oocytes with rBAT alone induced the uptake of L-cystine. Similar to the findings of other studies [36], this uptake was inhibited almost completely by the presence of L-arginine, suggesting that rBAT can interact with endogenous $b^{0,+}$AT-like components in the oocytes to induce system $b^{0,+}$-like transport activity. One could argue that the induction of L-cystine transport observed following the simultaneous injection of xCT and rBAT may be due solely to the expression of rBAT. Yet, the L-cystine transport observed following the injection of these two subunits was significantly greater than that measured following the injection of rBAT alone. Clearly, additional studies characterizing this uptake are necessary to understand fully this phenomenon.

Interestingly, it has been shown recently that system x_c^- is capable of transporting substrates other than those that have been traditionally associated with amino acid carriers. Two studies in cultured rat glioma cells (LRM55) have demonstrated that this carrier is capable of transporting xenobiotics that may induce cellular intoxication [37, 38]. These studies emphasize the importance of a mammalian model in which to study the activity of system x_c^-. Furthermore, as the kidney participates in the excretion of various toxic compounds, it is critical that the activity of various carriers and their substrate specificities are understood. The cell model described in this study can be used to provide a more complete understanding of the transport activities in renal tubular cells.

CONCLUSION

Data from the present study indicate that L-cystine and L-glutamate are high-affinity substrates for system x_c^- (when expressed in MDCK cells transfected stably with hxCT and h4F2hc). These data were validated by experiments using *Xenopus* oocytes, and thus, indicate that the xCT-4F2hc transfectants are reliable models for the study of system x_c^-. The development of a mammalian cell line in which system x_c^- is constitutively overexpressed is novel and highly relevant to the field of amino acid transport. To our knowledge, this study represents the first report of a mammalian renal cell line transfected stably with system x_c^-. This cell line can be used to further characterize the activity of system x_c^-.

ACKNOWLEDGMENTS

We thank Dr. Vadivel Ganapathy at the Medical College of Georgia, Augusta, Georgia, for kindly providing the clones for human xCT and human 4F2hc. We would also like to thank Dr. Puttur Prasad for helpful discussions and Ms. Jamie Battle for technical assistance. This work was supported, in part, by the National Institutes of Health (National Institute of Environmental Health Sciences) grants ES05980, ES05157, and ES11288 awarded to Dr. Zalups. Dr. Bridges is supported by a Ruth L. Kirschstein National Research Service Award, ES012556 awarded by the NIEHS.

Reprint requests to Dr. Rudolfs K. Zalups, Mercer University School of Medicine, Division of Basic Medical Sciences, 1550 College St., Macon, GA 31207.
E-mail: zalups_rk@mercer.edu

REFERENCES

1. Makowske M, Christensen HN: Contrasts in transport systems for anionic amino acids in hepatocytes and a hepatoma cell line HTC. *J Biol Chem* 257:5663–5670, 1982

2. Bannai S: Exchange of cystine and glutamate across plasma membrane of human fibroblasts. *J Biol Chem* 261:2256–2263, 1986
3. Bannai S, Kitamura EL: Transport interaction of L-cystine and L-glutamate in human diploid fibroblasts in culture. *J Biol Chem* 255:2372–2376, 1980
4. Bannai S, Kitamura E: Role of proton dissociation in the transport of cystine and glutamate in human diploid fibroblasts in culture. *J Biol Chem* 256:5770–5772, 1981
5. Foreman JW, Lee J, Segal S: Characteristics of cystine uptake by cultured LLC-PK1 cells. *Biochim Biophys Acta* 968:323–330, 1988
6. Takada A, Bannai S: Transport of cystine in isolated rat hepatocytes in primary culture. *J Biol Chem* 259:2441–2445, 1984
7. Sato H, Tamba M, Ishii T, Bannai S: Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. *J Biol Chem* 274:11455–11458, 1999
8. Sato H, Tamba M, Kuriyama-Matsumura K, *et al*: Molecular cloning and expression of human xCT, the light chain of amino acid transport system x_c^-. *Antiox Redox Signal* 2:665–671, 2000
9. Kim JY, Kanai Y, Chairoungdua A, *et al*: Human cystine/glutamate transporter: cDNA cloning and upregulation by oxidative stress in glioma cells. *Biochim Biophys Acta* 1512:335–344, 2001
10. Bridges CC, Kekuda R, Wang H, *et al*: Structure, function, and regulation of human cystine/glutatmate transporter in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* 42:47–54, 2001
11. Bassi MT, Gasol E, Manzoni M, *et al*: Identification and characterisation of human xCT that co-expresses, with 4F2 heavy chain, the amino acid transport activity system xc-. *Pflugers Arch* 442:286–296, 2001
12. Guidotti GG, Gazzola GC: Amino acid transporters: Systematic approach and principles of control, in *Mammalian Amino Acid Transport: Mechanisms and Control*, edited by Kilberg MS, Haussinger D, New York, Plenum, 1992, pp 3–30
13. Bannai S, Sato H, Ishii T, Taketani S: Enhancement of glutathione levels in mouse peritoneal macrophages by sodium arsenate, cadmium chloride, and glucose/glucose oxidase. *Biochim Biophys Acta* 1092:175–179, 1991
14. Bridges CC, Hu H, Miyauchi S, *et al*: Induction of cystine-glutamate transporter x_c^- by human immunodeficiency virus type 1 transactivator protein tat in retinal pigment epithelium. *Invest Ophthalmol Vis Sci* 45:2906–2914, 2004
15. Gukasyan HJ, Kannan R, Lee VHL, Kim K-J: Regulation of L-cystine transport and intracellular GSH level by a nitric oxide donor in primary cultured rabbit conjunctival epithelial cell layers. *Invest Ophthalmol Vis Sci*. 44:1202–1210, 2003
16. Miura K, Ishii T, Sugita Y, Bannai S: Cystine uptake and glutathione level in endothelial cells exposed to oxidative stress. *Am J Physiol* 262a:C50–C58, 1992
17. Ochi T: Arsenic compound-induced increases in glutathione levels in cultured Chinese hamster V79 cells and mechanisms associated with changes in gamma-glutamylcysteine synthetase activity, cystine uptake and utilization of cysteine. *Arch Toxicol* 71:730–740, 1997
18. Tomi M, Hosoya KI, Takanaga H, *et al*: Induction of xCT gene expression and L-cystine transport activity by diethyl maleate at the inner blood-retinal barrier. *Invest Ophthalmol Vis Sci* 43:774–779, 2002
19. Tomi M, Funaki T, Abukawa H, *et al*: Expression and regulation of L-cystine transporter, system xc-, in the newly developed rat retinal Muller cell line (TR-MUL). *Glia* 43:208–217, 2003
20. Watanabe H, Bannai S: Induction of cystine transport activity in mouse peritoneal macrophages. *J Exp Med* 165:628–640, 1987
21. Sato H, Nomura S, Maebara K, *et al*: Transcriptional control of cystine/glutamate transporter gene by amino acid deprivation. *Biochem Biophys Res Commun* 325:109–116, 2004
22. Bannai S, Ishii T: Transport of cystine and cysteine and cell growth in cultured human diploid fibroblasts: Effect of glutamate and homocysteate. *J Cell Physiol* 112:265–272, 1982
23. Bannai S: Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta* 779:289–306, 1984
24. Verrey F, Ristic Z, Romeo E, *et al*: Novel renal amino acid transporters. *Annu Rev Physiol* 67:557–572, 2005
25. Bauch C, Verrey F: Apical heterodimeric cystine and cationic amino acid transporter expressed in MDCK cells. *Am J Physiol* 283:F181–F189, 2002
26. Bridges CC, Zalups RK: Homocysteine, system $b^{0,+}$, and the renal epithelial transport and toxicity of inorganic mercury. *Am J Pathol* 165:1385–1394, 2004
27. Bridges CC, Bauch C, Verrey F, Zalups RK: Mercuric conjugates of cysteine are transported by the amino acid transporter, system $b^{0,+}$: Implications of molecular mimicry. *J Am Soc Nephrol* 15:663–673, 2004a
28. Cariappa R, Heath-Mornnig E, Furesz TC, *et al*: Stable polarized expression of hCAT-1 in an epithelial cell line. *J Memb Biol* 186:23–30, 2002
29. Palacín M, Estévez R, Bertran J, Zorzano A: Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* 78:969–1054, 1998
30. Aslamkhan AG, Han YH, Yang XP, *et al*: Human renal organic anion transporter 1-dependent uptake and toxicity of mercuric-thiol conjugates in Madin-Darby canine kidney cells. *Mol Pharmacol* 63:590–596, 2003
31. Palacín M, Fernández E, Chillarón J, Zorzano A: The amino acid transport system $b^{0,+}$ and cystinuria. *Mol Memb Biol* 18:21–26, 2001
32. Sato H, Kuriyama-Matsumura K, Siow RCM, *et al*: Induction of cystine transport via system x_c^- and maintenance of intracellular glutathione levels in pancreatic acinar and islet cell lines. *Biochim Biophys Acta* 1414:85–94, 1998
33. Boerner P, Evans-Layng M, Ü HS, Saier MH, Jr.: Polarity of neutral amino acid transport and characterization of a broad specificity transport activity in a kidney epithelial cell line, MDCK. *J Biol Chem* 261:13957–13962, 1986
34. Chillarón J, Roca R, Valencia A, *et al*: Heteromeric amino acid transporters: biochemistry, genetics, and physiology. *Am J Physiol Renal Physiol* 281:F995–F1018, 2001
35. Rajan DP, Kekuda R, Huang W, *et al*: Cloning and expression of a $b^{0,+}$-like amino acid transporter functioning as a heterodimer with 4F2hc instead of rBAT. *J Biol Chem* 274:29005–29010, 1999
36. Wang H, Tamba M, Kimata M, *et al*: Expression of the activity of cystine/glutamate exchange transporter, system x_c^-, by xCT and rBAT. *Biochem Biophys Res Commun* 305:611–618, 2003
37. Patel SA, Warren BA, Rhoderick JF, Bridges RJ: Differentiation of substrate and non-substrate inhibitors of transport system xc(-): An obligate exchanger of L-glutamate and L-cystine. *Neuropharmacol* 46:273–284, 2004
38. Warren BA, Patel SA, Nunn PA, Bridges RJ: The *Lathyrus* excitotoxin β-*N*-oxalyl-L-α,β-diaminopropionic acid is a substrate of the L-cystine/L-glutamate exchanger system x_c^-. *Toxicol Appl Pharmacol* 200:83–92, 2004