

# Structure, Function, and Regulation of Human Cystine/Glutamate Transporter in Retinal Pigment Epithelial Cells

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**PURPOSE.** The purpose of this investigation was to provide evidence for the expression of the cystine/glutamate transporter ( $x_c^-$ ) in the human retinal pigment epithelial cell line ARPE-19, clone the light chain of the transporter from an ARPE-19 cell cDNA library and study its function, and investigate the regulation of this transporter by nitric oxide (NO) in ARPE-19 cells.

**METHODS.** Uptake of radiolabeled cystine and glutamate was measured in ARPE-19 cells. The functional identity of  $x_c^-$  in these cells was established by substrate specificity and  $Na^+$ -independence of the uptake process. The human  $x_c^-$  light chain (human xCT) was cloned from an ARPE-19 cell cDNA library. The functional identity of the cloned human xCT was investigated by heterologous coexpression of the light chain with the heavy chain (human 4F2hc) in HeLa cells. ARPE-19 cells were treated with or without the NO donor 3-nitroso-*N*-acetylpenicillamine (SNAP) and the expression of  $x_c^-$  was studied at the functional and molecular levels.

**RESULTS.** ARPE-19 cells take up cystine as well as glutamate in the absence of  $Na^+$ . Substrate specificity studies indicate that although the uptake of cystine in the absence of  $Na^+$  is mediated by multiple amino acid transport systems including  $x_c^-$ , the uptake of glutamate in the absence of  $Na^+$  occurs exclusively via  $x_c^-$ . The human xCT cloned from ARPE-19 cells is a protein of 501 amino acids. These cells express the heavy chain 4F2hc as evidenced from RT-PCR analysis. Coexpression of human xCT with 4F2hc in HeLa cells leads to the induction of cystine and glutamate uptake with characteristics similar to that of  $x_c^-$ . The activity of  $x_c^-$  in ARPE-19 cells is upregulated by SNAP, and the process is associated with an increase in the expression of xCT with no detectable change in the expression of 4F2hc.

**CONCLUSIONS.** ARPE-19 cells express the cystine/glutamate transporter  $x_c^-$  (the light chain xCT and the heavy chain 4F2hc) as is evident from functional and molecular studies. NO upregulates this transport system and the process is associated with an increase in xCT mRNA but with no change in 4F2hc mRNA. (*Invest Ophthalmol Vis Sci.* 2001;42:47-54)

Glutathione plays an important role in several physiologic processes including protection of cells against oxidative damage.<sup>1-3</sup> Oxidative damage is thought to contribute to such

diseases as age-related macular degeneration<sup>4</sup> and diabetic retinopathy.<sup>5</sup> Glutathione has been detected in high concentrations in retina and retinal pigment epithelium (RPE).<sup>6,7</sup> The protective effects of exogenously administered glutathione against oxidative damage in cultured human RPE have been reported.<sup>8</sup> We have shown recently that glutathione protects the reduced-folate transporter in RPE cells from nitric oxide (NO)-induced loss of transport function.<sup>9</sup>

Glutathione is a tripeptide consisting of glutamic acid, cysteine, and glycine. Glutamate and glycine occur at relatively high intracellular concentrations; therefore, cysteine availability largely determines glutathione synthesis. The extracellular concentration of cysteine is quite low because this amino acid typically exists in the disulfide form, cystine.<sup>10</sup> To date, two different transport systems have been described that mediate the uptake of cystine in mammalian cells. They are  $b^{0,+}$  and  $x_c^-$ .<sup>11,12</sup> The expression of  $b^{0,+}$  is limited mostly to small intestine and kidney.  $b^{0,+}$  is a  $Na^+$ -independent transport system for cystine and also for a variety of neutral and cationic amino acids. This transport system exists as a heterodimer consisting of either rBAT (protein related to  $b^{0,+}$  amino acid transport system) or 4F2hc (heavy chain of the 4F2 cell surface antigen) as the heavy chain and  $b^{0,+}$  AT ( $b^{0,+}$  amino acid transporter) as the light chain.<sup>13-15</sup> In contrast to  $b^{0,+}$ , the system  $x_c^-$  is expressed ubiquitously, but at low levels, in mammalian tissues under normal conditions.<sup>16</sup> The expression of this system is upregulated, however, by oxidative stress.<sup>17-20</sup> Due to the widespread expression and regulation by oxidative stress, the system  $x_c^-$  is believed to be the primary transport system related to the uptake of cystine into cells for glutathione synthesis.  $x_c^-$  is  $Na^+$ -independent and mediates the entry of cystine into cells coupled to the efflux of glutamate.<sup>21</sup> Because of this obligatory exchange function,  $x_c^-$  is called cystine/glutamate transporter.  $x_c^-$  is also a heterodimer, consisting of 4F2hc as the heavy chain and xCT ( $x_c^-$  transporter) as the light chain.<sup>22</sup> 4F2hc is a subunit common to several amino acid transport systems including  $x_c^-$  and  $b^{0,+}$ , whereas xCT is unique to system  $x_c^-$ .<sup>11</sup> 4F2hc has been cloned from different animal species including humans.<sup>11</sup> In contrast, xCT has been characterized at the molecular and functional level only in mouse.<sup>22</sup>

The purpose of the present investigation was threefold: to provide evidence from functional studies for the expression of the transporter  $x_c^-$  in the human retinal pigment epithelial cell line ARPE-19; to clone the light chain of the transporter, xCT, from an ARPE-19 cell cDNA library and study its function in an heterologous expression system; and, to investigate the regulation of  $x_c^-$  by NO in ARPE-19 cells.

## MATERIALS AND METHODS

### Materials

[<sup>35</sup>S]-Cystine and [<sup>3</sup>H]-glutamate were purchased from New England Nuclear Corp. (Boston, MA). [<sup>3</sup>H]-Alanine and [<sup>3</sup>H]-arginine were purchased from Amersham Corp. (Piscataway, NJ). Cell culture supplies

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were purchased from Life Technologies (Gaithersburg, MD). Restriction enzymes were from New England Biolabs (Beverly, MA), 3-Nitroso-N-acetylpenicillamine (SNAP) was purchased from Research Biochemicals International (Natick, MA). Human retinal pigment epithelial cells (ARPE-19), a rapidly growing human RPE cell line<sup>23</sup> established in the laboratory of L. Hjelmeland (University of California, Davis), were provided by R. B. Caldwell (Medical College of Georgia, Augusta, GA). HeLa cells used for heterologous expression were purchased from American Type Culture Collection (Manassas, VA).

### Cell Culture

ARPE-19 cells were cultured in 75 cm<sup>2</sup> flasks with Dulbecco's modified Eagle's medium: nutrient mixture F12 (DMEM:F12), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cultures were passaged by dissociation in 0.05% (w/v) trypsin and seeded in 24-well culture plates (Fisher, Norcross, GA).

### Uptake Measurements in ARPE-19 Cells

For uptake experiments, the culture medium was removed from RPE cells and the cells were subsequently washed twice with uptake buffer, which lacked Na<sup>+</sup>. The composition of the uptake buffer was 25 mM 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES)/Tris, 140 mM *N*-methyl-D-glucamine chloride, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, and 5 mM glucose, pH 7.5. Uptake was initiated by adding 250 µl of uptake buffer containing radiolabeled substrates. Uptake measurements were done with incubations for a desired time at 37°C. At the end of the incubation, uptake was terminated by removal of the medium by aspiration followed by three washes with ice-cold uptake buffer without the radiolabeled substrates. The cells were then solubilized in 0.5 ml of 1% sodium dodecylsulfate in 0.2 N NaOH and transferred to scintillation vials for quantitation of radioactivity. This protocol is the same as described previously from our laboratory for the measurement of amino acid uptake in mammalian cells expressing heterologously the cloned amino acid transport proteins b<sup>0,+</sup>AT,<sup>13,15</sup> LAT1,<sup>24</sup> and LAT2.<sup>25</sup>

### RT-PCR for 4F2hc mRNA and Restriction Analysis of the RT-PCR Product

The x<sub>c</sub> system is a heterodimer consisting of 4F2hc as the heavy chain and xCT as the light chain. To determine whether ARPE-19 cells express 4F2hc mRNA, we used ARPE-19 cell mRNA for RT-PCR analysis with 4F2hc-specific primers. The sense primer was 5'-GCAGAAGTG-GTGGCACAC-3' corresponding to the nucleotide positions 451–468 in human 4F2hc cDNA,<sup>26</sup> and the antisense primer was 5'-TTTTGTAT-GCTCCCAGTAG AA-3' corresponding to the nucleotide positions 1033–1054 in human 4F2hc cDNA. The expected size of the RT-PCR product based on the nucleotide positions of the primer pair was 604 bp. The identity of the resulting product was established by restriction analysis. The product was gene cleaned and digested with *BalI* and *PstI*. The expected sizes of the digestion products were 335 and 269 for *BalI* and 412 and 192 for *PstI*.

### Construction of cDNA Library

The SuperScript plasmid system (Life Technologies) was used to establish a unidirectional cDNA library with poly(A)<sup>+</sup> mRNA isolated from ARPE-19 cells. Poly(A)<sup>+</sup> mRNA was prepared by subjecting total RNA twice to oligo(dT)-cellulose affinity chromatography before use in library construction. The cDNA products with sizes greater than 1 kbp were separated by size-fractionation and used for ligation at *SallI/NotI* site in pSPORT1 vector.

### cDNA Library Screening and DNA Sequencing

The ARPE-19 cell cDNA library was screened under low stringency conditions as described previously<sup>13,15,24,25</sup> using a mouse xCT cDNA fragment as the probe. The probe was prepared by RT-PCR using mouse lung mRNA and primers specific for mouse xCT. The primers 5'-TACCTGCAGGGCAATATG-3' (sense) and 5'-GGGCGTTTGTATC-

GAAGA-3' (antisense), corresponded to the nucleotide positions 362–379 and 1546–1563 in mouse xCT cDNA<sup>22</sup> and the expected size of the RT-PCR product was ~1.2 kbp. The product was subcloned in pGEM-T vector and sequenced for establishment of its identity. After the screening of the ARPE-19 cell cDNA library with the mouse xCT cDNA probe, positive clones were identified, and the colonies were purified by secondary screening. Both sense and antisense strands of the cDNA were sequenced using an automated Perkin-Elmer Applied Biosystems (Framingham, MA) 377 Prism DNA sequencer. The sequence was analyzed using the GCG sequence analysis software package, GCG version 10 (Genetics Computer Group, Inc., Madison, WI).

### Expression of the Cloned cDNA in HeLa Cells

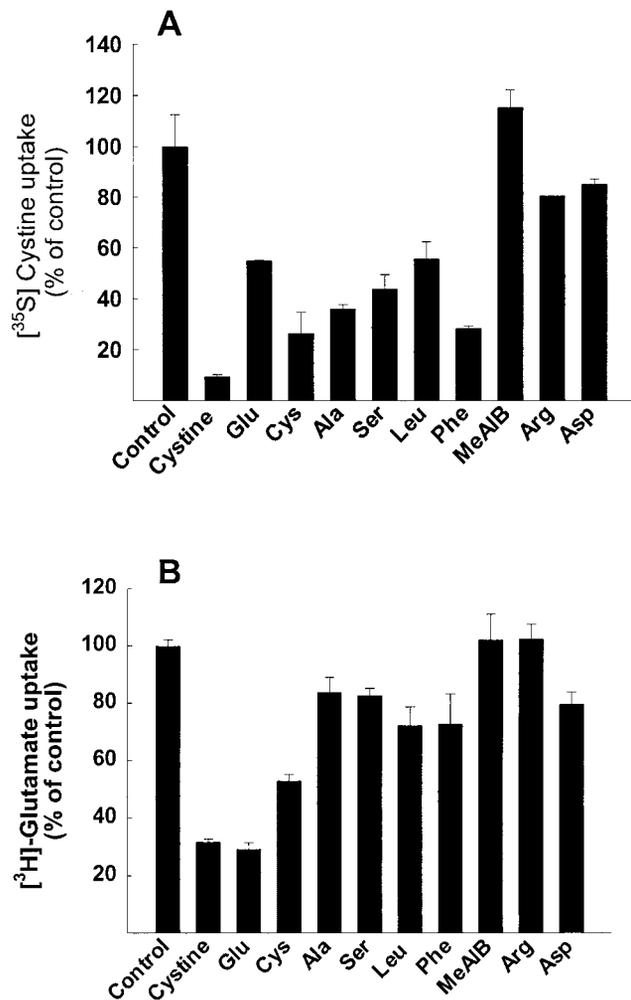
The vaccinia virus expression system was used to functionally characterize the cloned cDNA.<sup>13,15,24,25</sup> The human 4F2hc cDNA and the human xCT cDNA were cloned into pSPORT1 vector such that the sense transcription of the cDNA is under the control of T7 promoter. The human 4F2hc cDNA was isolated from a human placental choriocarcinoma cell (JAR) cDNA library and shown to be functional in heterologous expression systems.<sup>13,15,24,25</sup> The cDNAs were transfected into HeLa cells grown in 24-well tissue culture plates using Lipofectin, and the functional expression of the cDNAs was analyzed 12 hours later by measuring radiolabeled amino acid uptake. Transfection was done with human 4F2hc cDNA, human xCT cDNA or human 4F2hc cDNA plus human xCT cDNA. The transport buffer was composed of 25 mM Hepes/Tris (pH 7.5), supplemented with 140 mM *N*-methyl-D-glucamine chloride, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, and 5 mM glucose. The incubation time for the transport measurements was 15 minutes at 37°C, after which the uptake medium containing the radioactive substrate was aspirated off, and the cells were washed with 2 × 2 ml of ice-cold transport buffer. The cells were then solubilized in 0.5% SDS in 0.2 N NaOH, transferred to vials, and radioactivity associated with the cells quantitated by liquid scintillation spectrometry. The experiments were repeated two to three times with independent transfections, each done in duplicate or triplicate. Data are presented as means ± SE of these replicate measurements.

### Regulation Studies

To determine the effects of oxidative stress on the expression of the system x<sub>c</sub><sup>-</sup> in ARPE-19 cells, confluent cell cultures were treated with 1 mM SNAP, a NO-donor, at 37°C for 2, 4, or 24 hours. Cells treated similarly but in the absence of SNAP served as controls. After the treatment, uptake of radiolabeled substrates (cystine, glutamate, alanine, and arginine) into these cells was measured as described previously.

### Semiquantitative RT-PCR Analysis of the Steady-State Levels of mRNAs for xCT, 4F2hc and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

Confluent cultures of ARPE-19 cells were treated with or without 1 mM SNAP for 24 hours at 37°C and poly(A)<sup>+</sup> mRNA was then prepared from these cells. RT-PCR was carried out using primer pairs specific for human xCT, human 4F2hc, and human GAPDH. The primers specific for human xCT were 5'-AGGGGAGTCTCCATTATCAT-3' (sense) and 5'-AAGTAGGC CACATTTGTGTCAG-3' (antisense) corresponding to nucleotide positions 373–392 and 1078–1097 of the xCT cDNA cloned from ARPE-19 cell cDNA library. The primers specific for human 4F2hc were the same as described previously for the analysis of 4F2hc mRNA in ARPE-19 cells. The primers specific for human GAPDH were 5'-AAGGCTGAGAACGGGAAGCTTGTATCAAT-3' (sense) and 5'-TTC-CCGTTCCAGCTCAGGGATGACCTTGCCC-3' (antisense) corresponding to nucleotide positions 241–270 and 711–740 in human GAPDH cDNA.<sup>27</sup> Each of these RT-PCR products was subcloned in pGEM-T vector and sequenced to establish their identity. For semiquantitative RT-PCR, PCR following reverse transcription was carried out with varying numbers of cycles (range, 9 to 30). The products were size-fractionated on an agarose gel and subjected to Southern hybridization



**FIGURE 1.** Inhibition of  $\text{Na}^+$ -independent uptake of [ $^{35}\text{S}$ ]cystine (A) and [ $^3\text{H}$ ]glutamate (B) by various amino acids in cultured human ARPE-19 cells. Uptake of [ $^{35}\text{S}$ ]cystine (2.5  $\mu\text{M}$ ) and [ $^3\text{H}$ ]glutamate (2.5  $\mu\text{M}$ ) was measured in confluent ARPE-19 cells in a  $\text{Na}^+$ -free medium for 15 minutes at 37°C in the absence or presence of 2 mM unlabeled amino acids (except for cystine, 0.5 mM). Results are presented as percent of control uptake measured in the absence of unlabeled amino acids. The values for control uptake (100%) were  $46.8 \pm 5.8$  and  $7.46 \pm 0.27$  pmol/ $10^6$  cells/15 minutes for [ $^{35}\text{S}$ ] cystine and [ $^3\text{H}$ ] glutamate, respectively. Values represent means  $\pm$  SE for four determinations from two independent experiments.

with probes specific for each of the three products. These probes were generated by labeling the respective subcloned RT-PCR products with [ $^{32}\text{P}$ ]dCTP. The intensity of the hybridization signal was quantified using STORM phosphorimaging system (Molecular Dynamics, Sunnyvale, CA). The relationship between the intensity of the signal and the PCR cycle number was then analyzed to determine the linear range for the PCR product formation. The intensities of the signals within the linear range were used for data analysis.

## RESULTS

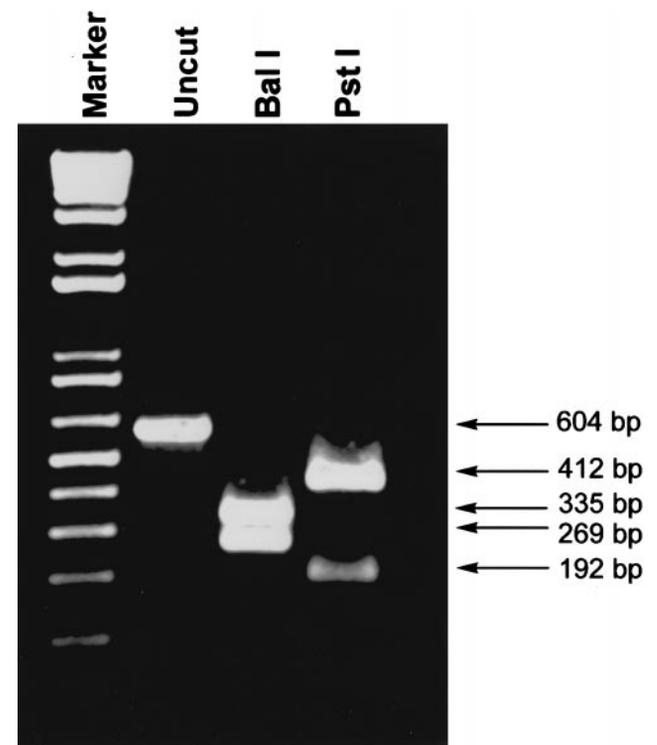
### Functional Evidence for the Expression of $\text{x}_c^-$ System in ARPE-19 Cells

To provide evidence for the expression of system  $\text{x}_c^-$  in ARPE-19 cells, the uptake of cystine and glutamate into these cells was studied in a  $\text{Na}^+$ -free medium and the substrate specificity of the uptake process assessed by competition experiments (Fig. 1). The cells were found to take up [ $^{35}\text{S}$ ]cystine and [ $^3\text{H}$ ]glutamate in the absence of  $\text{Na}^+$ , suggesting the pres-

ence of  $\text{Na}^+$ -independent transport mechanism(s) for these two amino acids in these cells. Competition experiments revealed that the  $\text{Na}^+$ -independent uptake of [ $^{35}\text{S}$ ]cystine was inhibitable by unlabeled cystine as well as by glutamate and a variety of neutral amino acids. In contrast, the  $\text{Na}^+$ -independent uptake of [ $^3\text{H}$ ]glutamate was inhibitable only by three amino acids, namely cystine, cysteine, and unlabeled glutamate. The substrate specificity of the transport system mediating the  $\text{Na}^+$ -independent uptake of glutamate in these cells matches with the known substrate specificity of system  $\text{x}_c^-$ . The inhibition of  $\text{Na}^+$ -independent cystine uptake by glutamate is clear evidence for the involvement of system  $\text{x}_c^-$  in cystine uptake. However, the substrate specificity of the cystine uptake process indicates that system  $\text{x}_c^-$  may not be the only transporter mediating the uptake of cystine in these cells. These data show that ARPE-19 cells express system  $\text{x}_c^-$  and that measurement of  $\text{Na}^+$ -independent glutamate uptake rather than cystine uptake identifies specifically the transport function of system  $\text{x}_c^-$  in these cells.

### Expression of 4F2 Heavy Chain in ARPE-19 Cells

System  $\text{x}_c^-$  is a heterodimer consisting of the 4F2 heavy chain (4F2hc) and the light chain  $\text{xCT}$ . If  $\text{x}_c^-$  is expressed in ARPE-19 cells, 4F2hc is expected to be expressed in these cells. Therefore, to provide evidence for the expression of 4F2hc mRNA in ARPE-19 cells, RT-PCR was performed with ARPE-19 cell mRNA and primers specific for human 4F2hc. An RT-PCR product of expected size (604 bp) was obtained from the mRNA, demonstrating the expression of 4F2hc mRNA in these cells (Fig. 2). The molecular identity of the resultant product was confirmed



**FIGURE 2.** RT-PCR and restriction analysis of 4F2hc mRNA in human ARPE-19 cells. Poly(A) $^+$  RNA from ARPE-19 cells was used for RT-PCR with human 4F2hc-specific primers. The expected size of the RT-PCR product (uncut), predicted from the positions of the primers, is 604 bp. The RT-PCR product was gene-cleaned and then used for restriction analysis with *Bal*I and *Pst*I. DNA standards (100 bp–12.2 kbp) were run on the gel in parallel to determine the size of the RT-PCR product and restriction fragments. The expected sizes of the digestion products are 335 and 269 bp for *Bal*I and 412 and 192 bp for *Pst*I.

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hxCT: 1  MVRKPVVSTISKGGYLGQNVNGRLPVLGNKEPPGQEKVQLKRKVTLLRGVSIITIGTIIGA 60
mxCT: 1  MVRKPVVATISKGGYLGQNMVSGRLPVMGDQEPGQEKVVLKKKITLLRGVSIITIGTVIGS 60
                                     I
hxCT: 61  GIFISPKGVLQNTGVSVMGLTIWTVCVGLSLFGALSYAELGTTIKKSGGHYTYILEVFGP 120
mxCT: 61  GIFISPKGILQNTGVSVMGLVFWVSACVGLSLFGALSYAELGTSIKKSGGHYTYILEVFGP 120
                                     II
hxCT: 121 LPAFVRVWVVELLIIRPAATAVISLAFGRYILEPFFIQCEIPELAIKLVITAVGITVVMVLN 180
mxCT: 121 LLAFVRVWVVELLVRPGATAVISLAFGRYILEPFFIQCEIPELAIKLVITAVGITVVMVLN 180
                                     III IV
hxCT: 181 SMSVSWASARIQIFLTFCKLTAILIIIVPGVMQLIKGQTONFKDAFSGRDSISITRLPLAFY 240
mxCT: 181 STSVSWASARIQIFLTFCKLTAILIIIVPGVIQLIKGQTHFKDAFSGRDTSLMGLPLAFY 240
                                     V
hxCT: 241 YGMAYAGWFYLFNFVTEEEVENPEKTIPLAICISMAIVTIGYVLTNVAYFTTINAEELLS 300
mxCT: 241 YGMAYAGWFYLFNFITEEVDNPEKTIPLAICISMAIITVGYVLTNVAYFTTISAEELLOS 300
                                     VI VII
hxCT: 301 NAVAVTFSEKRLGNFSLAVPIFVALSCFGSMNGGVFAVSRLEYVASREGHLPPELLSMIHV 360
mxCT: 301 SAVAVTFSEKRLGNFSLAVPIFVALSCFGSMNGGVFAVSRLEYVASREGHLPPELLSMIHV 360
                                     VIII
hxCT: 361 RKHTPLPAVIVLHPLTMIMLFSGDLDLNLNLFSEARWLFGLAVAGLIYLRKCPDMHRP 420
mxCT: 361 HKHTPLPAVIVLHPLTMVMLFSGDLYLLNLFSEARWLFGLAVAGLIYLRKCPDMHRP 420
                                     IX X
hxCT: 421 FKVELFIPALFSEFTCLFMVALSLYSDPFSTGIGFVITLTGVPAYYLFIIWDKPKRWFRRIM 480
mxCT: 421 FKVELFIPALFSEFTCLFMVALSLYSDPFSTGIGFVITLTGVPAYYLFIVWDKPKRWFRRIL 480
                                     XI XII
hxCT: 481 SEKITRTLQIILEVVPEDKL- 501
mxCT: 481 SDRITRTLQIILEVVPEDSKEL 502

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FIGURE 3. Amino acid sequence of human xCT cloned from ARPE-19 cells. The sequence of human xCT (hxCT) is aligned with the known sequence of mouse xCT (mxCT). Shaded boxes denote identical regions of the mouse and human xCT. Putative transmembrane domains are *underlined* and the *N*-linked glycosylation site in hxCT is indicated (Asn-314).

by restriction analysis with two different restriction enzymes (*BalI* and *PstI*). Both enzymes yielded restriction fragments of expected size from the RT-PCR product (Fig. 2).

### Primary Structure and Functional Characteristics of Human xCT Cloned from ARPE-19 Cells

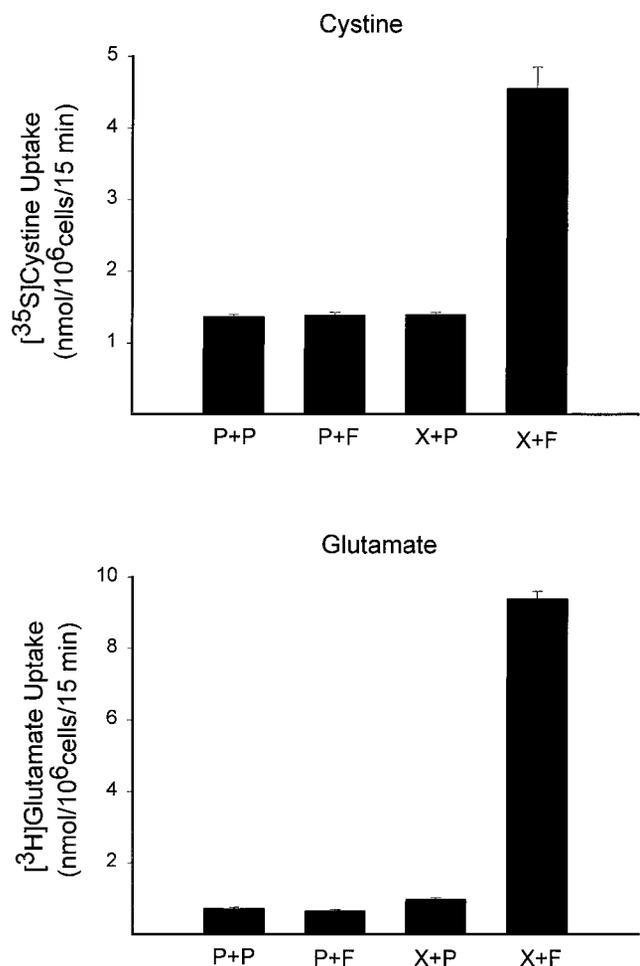
The light chain of system  $x_c^-$ , called xCT, has been cloned from mouse macrophage and its interaction with 4F2hc to form a transport-competent heterodimer has been demonstrated.<sup>22</sup> A GenBank database search revealed that the human homolog of xCT has been cloned and characterized at the structural level (GenBank accession no. AB026891). But, there is no information available in the literature on the functional characteristics of human xCT. To provide evidence for the expression of xCT mRNA in these cells and to elucidate the functional characteristics of the xCT protein, we cloned the full-length xCT cDNA from these cells. This was accomplished by constructing an ARPE-19 cell cDNA library followed by screening of the library with a mouse xCT-specific cDNA fragment as the probe. The human xCT cDNA isolated from the library is 2482 bp-long with a poly(A) tail (GenBank accession no. AF252872). This cDNA has a coding region of 1506 bp (including the termination codon), flanked by a 231 bp-long 5'-untranslated region and a 746 bp-long 3'-untranslated region. The cDNA cloned from ARPE-19 cells is considerably longer than the cDNA reported in the GenBank, which is 1852 bp-long. The difference between the two cDNAs is in the 3'-untranslated region. Because both cDNAs possess a poly(A) tail, the difference in the 3'-untranslated region arises most likely from alternative splicing. Both cDNAs however code for an identical protein of 501 amino acids with 12 putative transmembrane domains. The amino acid sequence of human xCT is highly homologous to that of mouse xCT with 89% identity and 96% similarity (Fig. 3). One interesting difference between the human and mouse xCT proteins however is that human xCT contains a potential site for *N*-linked glycosylation (Asn-314) in the extracellular loop between transmembrane domains 7 and 8, whereas mouse xCT does not possess any potential site for *N*-linked glycosylation. This raises the possibility that human xCT, but not mouse xCT, may be a glycoprotein.

Functional characteristics of the human xCT cloned from ARPE-19 cells were analyzed by heterologous expression of the cDNA in HeLa cells. Human xCT cDNA was expressed either alone or with human 4F2hc cDNA and the transport function was monitored by measuring the uptake of cystine and glutamate in the absence of  $\text{Na}^+$  (Fig. 4). The uptake of these amino acids in HeLa cells transfected with vector alone was similar to the uptake in HeLa cells expressing 4F2hc or xCT independently. This shows that neither 4F2hc nor xCT, when expressed alone, is capable of amino acid transport. However, when 4F2hc and xCT were coexpressed, the uptake of cystine and glutamate increased several-fold. These results show that the 4F2hc/xCT heteromeric complex is capable of mediating  $\text{Na}^+$ -independent uptake of cystine and glutamate.

The substrate specificity of the transport process associated with the 4F2hc/xCT complex was investigated by assessing the ability of various amino acids to compete with [ $^3\text{H}$ ]glutamate for  $\text{Na}^+$ -independent uptake into HeLa cells coexpressing human 4F2hc and human xCT heterologously (Fig. 5A). Unlabeled glutamate and cystine were the most potent inhibitors of [ $^3\text{H}$ ]glutamate uptake mediated by the heteromeric complex. Cysteine and aspartate also showed appreciable inhibition. Other amino acids including various neutral amino acids and the cationic amino acid arginine exhibited small or no inhibitory potency. The uptake of glutamate mediated by the 4F2hc/xCT complex was saturable over a glutamate concentration range of 0.025 to 2 mM (Fig. 5B). The uptake data conformed to the Michaelis-Menten kinetics describing a single saturable transport system. The apparent Michaelis-Menten constant ( $K_m$ ) and the maximal velocity ( $V_{max}$ ) for the uptake process were  $48 \mu\text{M} \pm 4 \mu\text{M}$  and  $25.5 \pm 0.4 \text{ nmol}/10^6 \text{ cells}/15 \text{ min}$ , respectively.

### Regulation of Expression of System $x_c^-$ by NO in ARPE-19 Cells

The regulation of system  $x_c^-$  function by NO in ARPE-19 cells was investigated at the functional level as well as at the molecular level. Treatment of confluent cultures of ARPE-19 cells with SNAP, a NO donor, for 24 hours increased the  $\text{Na}^+$ -independent uptake of cystine and glutamate (Fig. 6). This

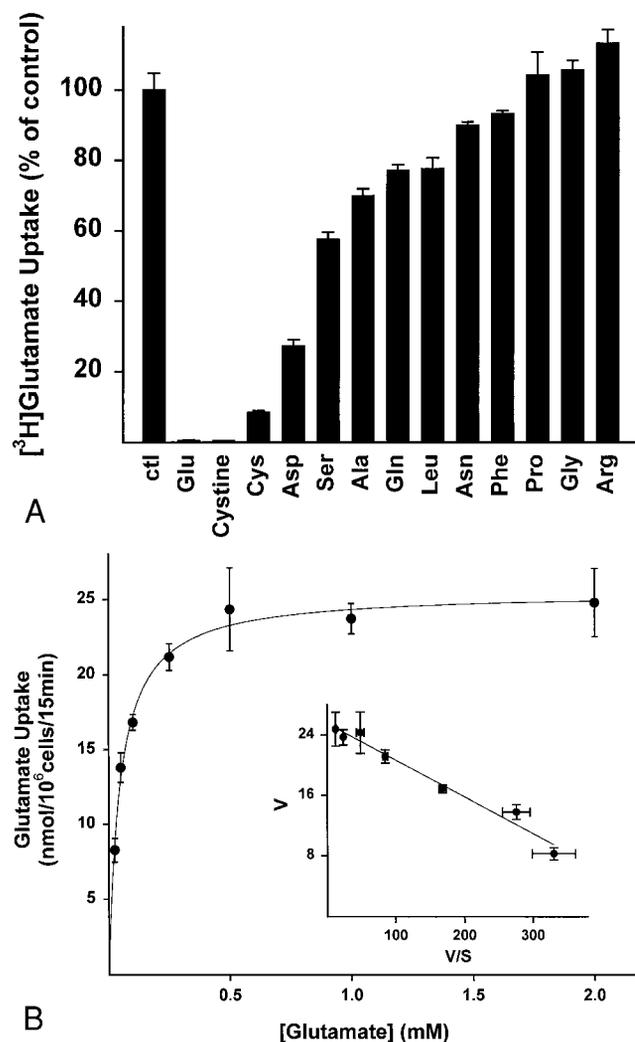


**FIGURE 4.** Transport function of the 4F2hc/xCT complex in HeLa cells. Cells were transfected with pSPORT vector (P + P), h4F2hc cDNA (P + F), hxCT cDNA (P + X) or hxCT cDNA plus h4F2hc cDNA (X + F). DNA content during transfection was kept constant by the addition of the pSPORT vector (P) as required. Expression of the transfected cDNAs was carried out by the vaccinia virus expression technique. Uptake of [<sup>35</sup>S]cystine (25  $\mu$ M) and [<sup>3</sup>H]glutamate (25  $\mu$ M) was measured in these cells in a Na<sup>+</sup>-free medium for 15 minutes at 37°C. Values are means  $\pm$  SE for six determinations done with two independent transfections.

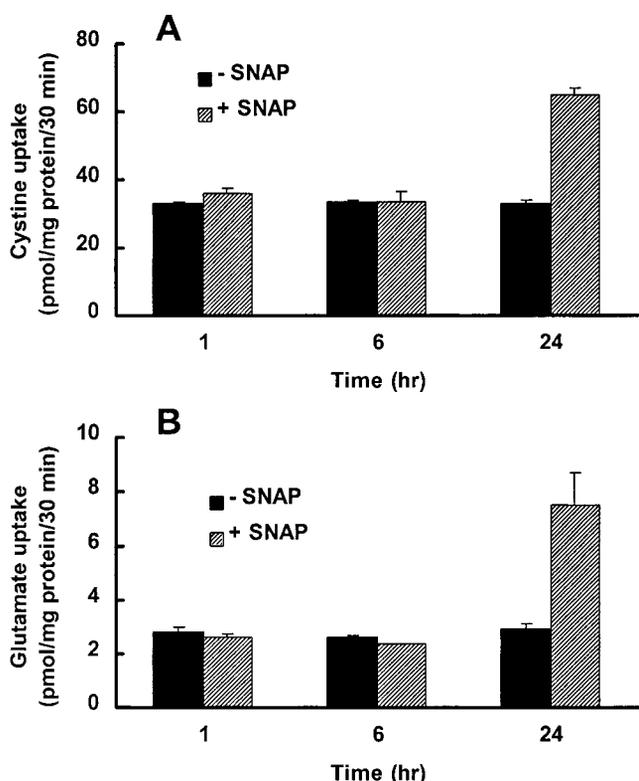
increase was not observed when the treatment time was 1 hour or 6 hours. The stimulatory effect of SNAP on the uptake of cystine and glutamate was specific because under identical conditions the treatment did not increase the Na<sup>+</sup>-independent uptake of two other amino acids, namely alanine and arginine (data not shown). System x<sub>c</sub><sup>-</sup> does not accept alanine or arginine as substrates. In contrast, the Na<sup>+</sup>-independent glutamate uptake is entirely due to the activity of system x<sub>c</sub><sup>-</sup>. These results therefore show that NO induces the activity of system x<sub>c</sub><sup>-</sup> specifically. We then analyzed the kinetics of system x<sub>c</sub><sup>-</sup> activity in control cells and in cells treated with SNAP (Fig. 7). The transport function of system x<sub>c</sub><sup>-</sup> was monitored by measuring the Na<sup>+</sup>-independent uptake of glutamate. The kinetic studies with glutamate as the substrate showed that the increase in the transport activity of system x<sub>c</sub><sup>-</sup> observed in SNAP-treated ARPE-19 cells compared to control cells was primarily associated with an increase in the maximal velocity of the transporter with no significant change in the substrate affinity. The maximal velocity of glutamate uptake was 2.7-fold greater in SNAP-treated cells than in control cells (32.3  $\pm$  0.4 vs. 11.8  $\pm$  0.7 nmol/mg of protein/30 min). The Michaelis-Menten constant for glutamate remained almost the same in

SNAP-treated cells and in control cells (221  $\pm$  18  $\mu$ M vs. 275  $\pm$  23  $\mu$ M).

Next examined was the influence of cycloheximide (an inhibitor of translation) and actinomycin D (an inhibitor of transcription) on SNAP-induced increase in x<sub>c</sub><sup>-</sup> activity. Both compounds decreased the stimulatory effect of SNAP to a significant extent. The stimulation of x<sub>c</sub><sup>-</sup> activity by SNAP in the absence of inhibitors was 2.7-fold  $\pm$  0.1-fold. This value decreased to 2.0  $\pm$  0.1 in the presence of cycloheximide (75  $\mu$ g/ml) and 1.3  $\pm$  0.1 in the presence of actinomycin D (7.5



**FIGURE 5.** Substrate specificity (A) and saturation kinetics (B) of the transport function induced by the h4F2hc/hxCT complex in HeLa cells. Cells were transfected with either pSPORT alone or h4F2hc cDNA plus hxCT cDNA and the transfected cDNAs were expressed by the vaccinia virus expression technique. For the substrate specificity studies, uptake of [<sup>3</sup>H]glutamate (25  $\mu$ M) in these cells was measured in a Na<sup>+</sup>-free medium for 15 minutes at 37°C in the absence or presence of 5 mM various amino acids. Data represent only the h4F2hc/hxCT complex-specific uptake (i.e., uptake in cells transfected with h4F2hc cDNA plus hxCT cDNA minus uptake in cells transfected with vector alone) and are given as percent of control uptake measured in the absence of unlabeled amino acids (100% = 11.0  $\pm$  0.5 nmol/10<sup>6</sup> cells/15 min). Values are means  $\pm$  SE for six determinations from two independent transfections. For saturation kinetics studies, uptake of glutamate was measured in a Na<sup>+</sup>-free medium for 15 minutes at 37°C over a glutamate concentration range of 0.025 to 2 mM. Data represent only the h4F2hc/hxCT complex-specific uptake. Values are means  $\pm$  SE for six determinations from two separate transfections. *Inset:* Eadie-Hofstee plot.



**FIGURE 6.** Upregulation of the  $\text{Na}^+$ -independent uptake of cystine (A) and glutamate (B) in ARPE-19 cells by SNAP. Confluent cells were treated in the absence or presence of 1 mM SNAP at  $37^\circ\text{C}$  for 1, 6, or 24 hours. Uptake of [ $^{35}\text{S}$ ]cystine ( $2.5 \mu\text{M}$ ) and [ $^3\text{H}$ ]glutamate ( $0.2 \mu\text{M}$ ) was measured in these cells for 30 minutes at  $37^\circ\text{C}$  in a  $\text{Na}^+$ -free medium. Values are means  $\pm$  SE for six determinations from two independent experiments.

$\mu\text{g/ml}$ ). These data suggest that the stimulatory effect of SNAP on  $x_c^-$  activity involves de novo synthesis of  $x_c^-$ .

The influence of the SNAP treatment was studied on the steady-state levels of mRNA transcripts specific for the two protein components of system  $x_c^-$ , namely 4F2hc and xCT. mRNA samples isolated from control and SNAP-treated ARPE-19 cells were used for semiquantitative RT-PCR for the determination of the levels of mRNA transcripts. As an internal control, the steady-state levels of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined in the mRNA samples in parallel. The results of these experiments showed that treatment with SNAP did not alter the steady-state levels of 4F2hc mRNA (Fig. 8). The small increase in 4F2hc mRNA seen in SNAP-treated cells versus control cells was similar to the increase in the steady-state levels of GAPDH mRNA. In contrast, the steady-state levels of xCT mRNA increased markedly in SNAP-treated cells compared to control cells and this increase was much higher than observed in the case of 4F2hc mRNA and GAPDH mRNA. These results demonstrate that the SNAP-induced increase in the transport activity of system  $x_c^-$  is associated with an increase in the expression of xCT with no significant change in the expression of 4F2hc.

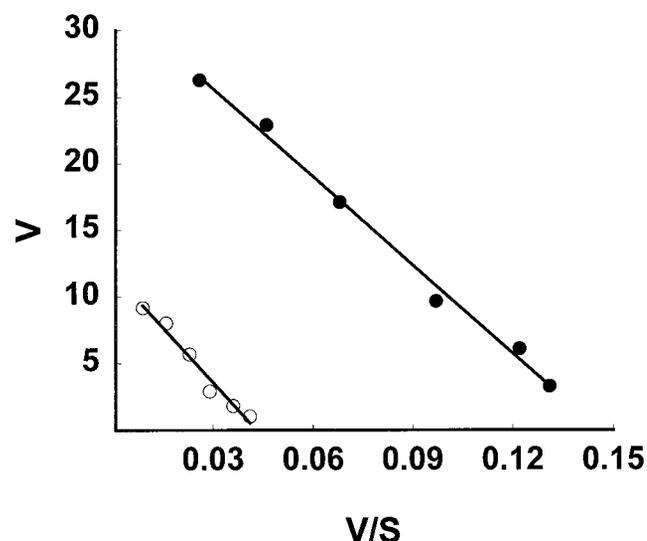
## DISCUSSION

As the cellular uptake of cystine is responsible for the availability of cysteine for intracellular glutathione synthesis, studies of the expression and regulation of the transport systems that mediate this uptake process in ocular tissues are of physiological and clinical significance. The eye is exposed constantly to

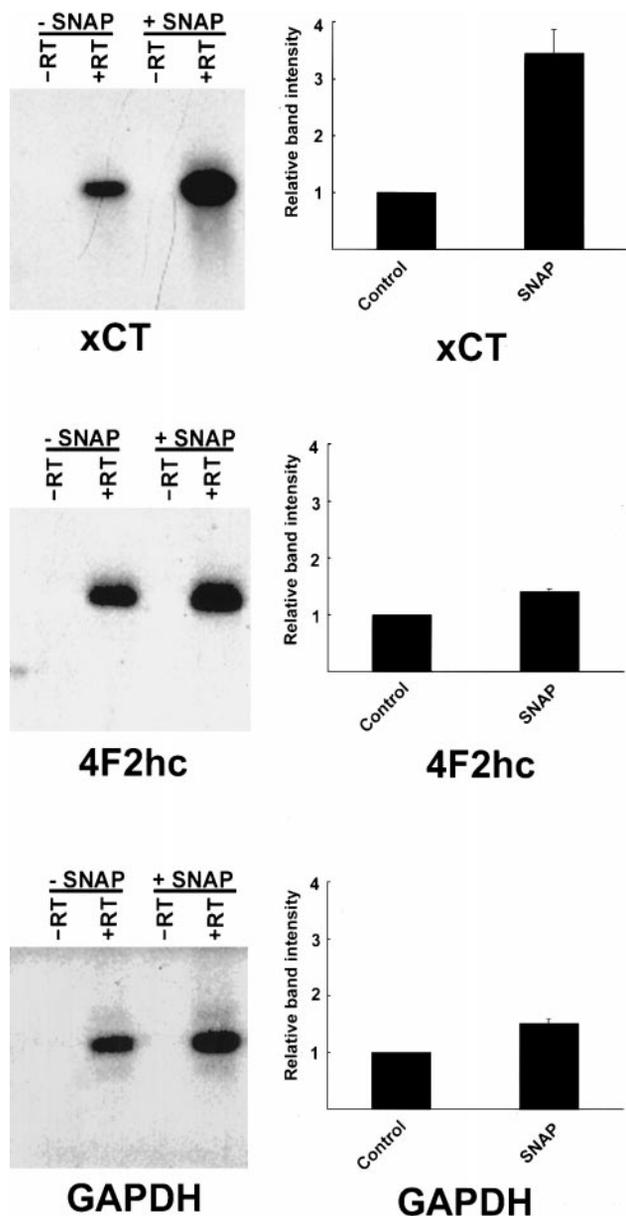
irradiation; therefore, the ocular tissues have an extraordinary need for antioxidant protection. Glutathione in these tissues may serve to provide this protection. The present investigation was undertaken to examine the expression of the amino acid transport system  $x_c^-$  in RPE cells because system  $x_c^-$  plays a primary role in supplying cysteine for glutathione synthesis, and this system is regulated by oxidative stress.<sup>17-20</sup> This investigation has provided clear evidence at the functional and molecular level for the expression of system  $x_c^-$  in these cells and also for the regulation of its expression by NO.

System  $x_c^-$  is a  $\text{Na}^+$ -independent exchanger for cystine and glutamate and couples the influx of cystine to the efflux of glutamate in cells. This system can be studied by monitoring the  $\text{Na}^+$ -independent uptake of radiolabeled cystine or glutamate, which occurs in exchange with intracellular glutamate. Functional studies in ARPE-19 cells using this approach demonstrate that system  $x_c^-$  is expressed in these cells. In addition to system  $x_c^-$ , other transport systems such as  $\text{b}^{0,+}$  may also be present in these cells that contribute to the influx of cystine. However, when glutamate is used as the substrate instead of cystine, the uptake appears to be mediated exclusively by system  $x_c^-$ .

System  $x_c^-$  functions as a heterodimer consisting of 4F2hc and xCT. Evidence is shown here for the expression of both of these components in ARPE-19 cells. The presence of 4F2hc mRNA in these cells was established by RT-PCR. The presence of xCT mRNA was shown by isolation of a full-length xCT cDNA from an ARPE-19 cell cDNA library. Even though the nucleotide sequence of human xCT cDNA has been deposited in the GenBank database by other investigators (accession number AB026891), no information is available on the functional identity of the clone. In this article, evidence is provided for the first time for the transport function of the cloned human xCT. This was accomplished by using a heterologous expression system in which human 4F2hc and human xCT were coexpressed. If expressed independently, neither of these proteins exhibits detectable transport function. However, when coexpressed, the 4F2hc/xCT heteromeric complex mediates the transport of cystine and glutamate. The functional



**FIGURE 7.** Kinetics of glutamate uptake in control (○) and SNAP-treated (●) ARPE-19 cells. Confluent cells were treated with or without 1 mM SNAP for 24 hours at  $37^\circ\text{C}$ . Uptake of glutamate was measured in these cells for 30 minutes at  $37^\circ\text{C}$  in a  $\text{Na}^+$ -free medium over a glutamate concentration range of  $25 \mu\text{M}$  to  $1000 \mu\text{M}$ . Values are means  $\pm$  SE for six determinations from two independent experiments. Results are presented as Eadie-Hofstee plots (V, glutamate uptake in nmol/mg of protein/30 min; S, glutamate concentration in  $\mu\text{M}$ ).



**FIGURE 8.** Analysis of steady-state levels of mRNA for xCT, 4F2hc, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in control (– SNAP) and SNAP-treated (+ SNAP) ARPE-19 cells. Confluent cells were treated with or without 1 mM SNAP for 24 hours at 37°C. Poly(A)<sup>+</sup> RNA was then isolated from these cells and used for semi-quantitative RT-PCR. Primer pairs specific for human xCT mRNA, human 4F2hc mRNA and human GAPDH mRNA were used. RT-PCR was done with a wide range of PCR cycles (9–30). The resultant products were run on a gel and then subjected to Southern hybridization with <sup>32</sup>P-labeled cDNA probes specific for xCT, 4F2hc, and GAPDH. The hybridization signals were quantified using the STORM Phosphorimaging System, and the intensities that were in the linear range with the PCR cycle number were used for analysis. *Left:* representative Southern hybridization signal. *Right:* band intensity in SNAP-treated cells relative to band intensity in control cells.

characteristics of the transport process associated with the heteromeric complex are similar to those of system x<sub>c</sub><sup>−</sup>. It is interesting that the substrate affinity of the heterologously expressed system x<sub>c</sub><sup>−</sup> is significantly different from that of the constitutively expressed system x<sub>c</sub><sup>−</sup> (K<sub>t</sub> value: 48 ± 4 vs. 221 ± 18 μM). This difference is likely due to the different cell types used in the determination of the substrate affinity. The heterologously expressed system x<sub>c</sub><sup>−</sup> was studied in HeLa cells,

whereas the constitutively expressed system x<sub>c</sub><sup>−</sup> was studied in ARPE-19 cells. The kinetic parameters of a transport system may differ significantly in different cell types, influenced by the cell type-specific differences in posttranslational modification such as glycosylation and in the microenvironment of the transport protein in the membrane.

After establishing the expression of system x<sub>c</sub><sup>−</sup> in ARPE-19 cells at the functional and molecular level, we investigated the regulation of the expression of this transport system by NO. Exposure of the cells to SNAP, an NO donor, for 24 hours leads to an increase in the Na<sup>+</sup>-independent uptake of cystine and glutamate in these cells. This stimulatory effect is not seen if the exposure time is less than 6 hours. The increase in system x<sub>c</sub><sup>−</sup> activity resulting from NO-exposure is accompanied by an increase in the maximal velocity of the transport process. The substrate affinity of the process is not altered. These results, together with the need for a long treatment period to elicit the observed effect, suggest that the increase in transport function is most likely due to an increase in the transporter density in the cell plasma membrane. This is supported by the findings that cycloheximide (an inhibitor of translation) and actinomycin D (an inhibitor of transcription) attenuate the stimulatory effect of SNAP to a significant extent. As system x<sub>c</sub><sup>−</sup> is a heterodimer consisting of 4F2hc as the heavy chain and xCT as the light chain, the question arises as to which of these two proteins is expressed at higher levels as a result of exposure to NO. We addressed this question by quantifying the steady-state levels of 4F2hc mRNA and xCT mRNA in control and NO-treated cells. These studies have shown that NO increases only the steady-state levels of xCT mRNA. The levels of 4F2hc mRNA are not influenced by NO. The specific stimulatory effect of NO on xCT expression suggests that the levels of xCT constitute the rate-limiting factor in the formation of the transport-competent 4F2hc/xCT heteromeric complex. Furthermore, 4F2hc is not unique to system x<sub>c</sub><sup>−</sup> because this protein is also a component of several other amino acid transport systems such as systems L, y<sup>+</sup>L, and b<sup>0+</sup>. Therefore, the stimulatory influence of NO on the expression of only xCT confers the specificity of NO action on system x<sub>c</sub><sup>−</sup>.

The stimulation of x<sub>c</sub><sup>−</sup> expression by NO has physiological relevance. Oxidative stress is expected to deplete the cellular levels of antioxidants such as glutathione. An increase in the expression of system x<sub>c</sub><sup>−</sup> following exposure to NO may be a physiologic response of the cell to oxidative stress as an attempt to increase the cellular levels of cysteine for glutathione synthesis. Although studies from other laboratories have demonstrated the stimulatory effect of oxidative stress on system x<sub>c</sub><sup>−</sup> in different cell types,<sup>17–20</sup> for the first time here, the basis for the stimulatory effect at the molecular level is described. Several diseases in humans are associated with oxidative stress. These include diabetes, bacterial infection, AIDS, and several neurodegenerative disorders. It is speculated that the expression of system x<sub>c</sub><sup>−</sup> may be significantly upregulated under these pathologic conditions in an attempt to increase glutathione levels as an antioxidant protective mechanism against the oxidative stress.

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