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xCT Cystine Transporter Expression in HEK293 Cells: Pharmacology and Localization

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xCT, the core subunit of the system x_c^- high affinity cystine transporter, belongs to a superfamily of glycoprotein-associated amino acid transporters. Although xCT was shown to promote cystine transport in *Xenopus* oocytes, little work has been done with mammalian cells (Sato, H., Tamba, M., Ishii, T., and Bannai, S. *J. Biol. Chem.* 274, 11455–11458, 1999). Therefore, we have constructed mammalian expression vectors for murine xCT and its accessory subunit 4F2hc and transfected them into HEK293 cells. We report that this transporter binds cystine with high affinity (81 μM) and displays a pharmacological profile expected for system x_c^-. Surprisingly, xCT transport activity in HEK293 cells is not dependent on the coexpression of the exogenous 4F2hc. Expression of GFP-tagged xCT indicated a highly clustered plasma membrane and intracellular distribution suggesting the presence of subcellular domains associated with combating oxidative stress. Our results indicate that HEK293 cells transfected with the xCT subunit would be a useful vehicle for future structure-function and pharmacology experiments involving system x_c^-.

Key Words: **system x_c^-; xCT; cystine transporter; glutamate; oxidative stress.**

The recently discovered glycoprotein-associated amino acid transporters (gpAATs) constitute a distinct family within the amino acid (a.a.)/polyamine/choline superfamily of transporters (2, 3). Each gpAAT function as a heterodimeric transmembrane protein consisting of a highly glycosylated heavy chain (4F2hc/CD98 or rBAT) that is common to all gpAATs, and a light chain which confers transporter substrate specificity. Currently, five vertebrate gpAATs have been shown to associate with 4F2hc/CD98: LAT1 (4), LAT2 (5), y^+LAT1 (6), y^+LAT2 (7), and xCT (1). These transporters are Na^+-independent obligatory a.a. antiporters with broad substrate specificity, with the exception of xCT (discussed below). LAT(1/2) exchanges large neutral a.a. (e.g., leucine) and y^+LAT(1/2) exchanges cationic a.a. for large neutral a.a. cotransported with Na^+.

The subunit xCT is a recent addition to this family, having been cloned from mouse activated macrophages by expression in *Xenopus* oocytes (1). xCT is unique in its Na^+-independence and its high affinity antiport exchange of cystine and glutamate. In association with 4F2hc, xCT has been recognized as a candidate protein for transport system x_c^-, a system that describes an exchange agency specific for anionic amino acids, particularly cystine and glutamate, in the plasma membrane of cultured mammalian cells (1, 8–15). Our laboratory has previously characterized system x_c^- activity in the N18-RE-105 neuronal cell line and rat immature cortical neurons in the context of glutamate toxicity due to inhibition of cystine uptake leading to glutathione depletion and oxidative stress (15, 16).

The identification of cDNAs encoding a cystine-glutamate antiport system may now provide the means to manipulate cystine transport into mammalian cells (1). Accordingly, as a first step we have proceeded to characterize the xCT subunit function in human embryonic kidney cells (HEK293). Here we report that xCT expression in HEK293 is not limited by the availability of the accessory subunit 4F2hc. We also show that the pharmacology of xCT-mediated transport agrees with that of system x_c^- transport characterized previously (1, 13, 15–18). Finally, we demonstrate that a GFP (green fluorescent protein)-tagged version of xCT localizes with a distinctly clustered distribution in HEK293 cells. These clusters may represent subcellular sites responsible for the combat of oxidative stress.

METHODS

Cell culture and transfection. Human embryonic kidney 293 cells (HEK293) (ATCC) were plated on 60 mm culture dishes (Falcon) and maintained at 37°C in a humidified 95% O_2–5% CO_2 incubator. Culture medium was prepared from minimal essential medium (Life

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Technologies) supplemented with 10% fetal bovine serum (Hyclone), 1 mM sodium pyruvate (Life Technologies), 100 U/mL penicillin-streptomycin (Life Technologies), and 1 mM glutamine (Sigma). Cells were transiently transfected using the calcium-phosphate method (19) when at 60–70% confluency. For transfection, the cDNA plasmid ratio for xCT, 4F2hc, and pBluescript (Stratagene) was varied accordingly (see Results) with a total amount of 2.5 μg DNA/60 mm plate. Cells were transfected in a humidified 97% O_2–3% CO_2 atmosphere at 37°C for ~8 h and then split among the wells of a poly-D-lysine-coated (Sigma) 24-well plate (Costar) at a density of ~4000 cells per well in 0.5 mL media. To facilitate detachment of the cells during the split into a 24-well plate, the transfection protocol was modified to include a 5-min incubation with 3 mL/60 mm plate of a Ca^{2+} and Mg^{2+} free D1 solution consisting of 5 mM KCl, 138 mM NaCl, 4 mM $NaHCO_3$, 5.6 mM D-glucose, and 0.01 mM phenol red. Cells were allowed to express transfected proteins for ~36 h before L-[^{3}H]-glutamate uptake assays were performed.

Generation of xCT and 4F2hc cDNA mammalian expression constructs. The mouse isoforms of xCT and 4F2hc (a gift from S. Bannai, University of Tsukuba, Japan) (GenBank Accession No. AB022345 and X14309, respectively) were excised from a prokaryotic expression vector (pSport) and subcloned into the mammalian expression vector pcDNA3.1+ (Invitrogen). xCT was subcloned into the *Not*I/*Eco*RI sites and 4F2hc into the *Not*I/*Kpn*I sites. To ensure proper initiation of translation with the xCT construct, a potential start codon 312 bp upstream of the true Kozak contained start codon in the 5′ untranslated region was removed by excision with *Eco*RI and *Sac*II (New England Biolabs, NEB). The DNA ends were then blunted using DNA polymerase I large Klenow fragment (NEB) and ligated with T4 DNA ligase (NEB).

Plasmids were transformed and amplified in *E. coli* DH5α (Gibco BRL) and harvested using a Qiagen Maxiprep kit according to the manufacturer's instructions. An upstream CMV promotor drove all plasmids used. The sequences of both xCT and 4F2hc mammalian expression vectors were verified by DNA sequencing (NAPS sequencing unit, UBC).

Construction of xCT-GFP fusion vector. The xCT cDNA was amplified from a prokaryotic expression vector (pSport) using PCR primers (forward primer, 5′-AAGCTTGCCGCCATGGTCAGAAA-GCCAGTT-3′) and (reverse primer, 5′-GGTACCTAATTCTTTA-GAGTCTTC-3′). The ~1.5 kb PCR product was cloned into a TA cloning vector (pCR2.1, Invitrogen). The PCR product was then excised from pCR2.1 using *Hin*dIII (NEB) and *Kpn*I (NEB) and subcloned into the GW1-CMV, a construct previously used for creating C-terminal GFP fusion proteins (20). The final construct was transfected into HEK293 cells using the calcium-phosphate method and allowed to express transfected proteins for 36–72 h. The cells were then viewed under wide field or confocal fluorescence microscopy.

L-[^{3}H]-glutamate uptake assays in HEK293 cultures. All chemicals were from Sigma unless otherwise stated. We have previously reported the dose-inhibition and pharmacological characteristics of L-[^{35}S]-cystine uptake by endogenous system x_c^- in N18-RE-105 cells (15). However, due to the unavailability of reasonably priced radiolabeled cystine we have performed uptake experiments with L-[^{3}H]-glutamate which is an equally well-characterized substrate for system x_c^- (1, 15–18). For uptake assays, medium was removed from each well by aspiration, and the adherent cells were washed once with 2 mL/well of 37°C Na^+-free Hank's balanced salt solution (HBSS) consisting of 140 mM *N*-methyl-D-glucamine, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM KH_2PO_4, 10 mM Hepes, 5 mM D-glucose (pH 7.4). The cells were preincubated for 20 min at 37°C with an additional 500 μL of Na^+-free HBSS, then washed again as described above. The medium was removed by aspiration and replaced with 300 μL of Na^+-free HBSS containing (38 nM) L-[^{3}H]-glutamate (42 Ci/mmol) (Amersham Pharmacia) and the indicated amounts of unlabeled compounds: L-glutamate, L-cystine, quisqualate, L-homocysteate, D-aspartate, L-aspartate, and kainate. After the cells were incubated with the radiolabel for 20 min at 37°C, the medium was removed and the cells were washed rapidly three times with 2 mL of ice cold Na^+-free HBSS, without added amino acids. The cells were lysed with 200 μL of 0.1 M potassium phosphate buffer (pH 7.0) containing 0.5% (v/v) Triton X-100. One hundred microliters of the lysate was removed for radioactivity determination by liquid scintillation counting in an aqueous accepting fluor. The remaining 100 μL was retained for subsequent protein determination using a BCA protein assay kit (Pierce).

RESULTS AND DISCUSSION

To determine whether functional system x_c^- transport activity could be over-expressed in mammalian cells, we transiently transfected HEK293 cells with either control DNA (pBluescript), 4F2hc vector alone, or different amounts of 4F2hc vector with a constant level of xCT vector. In these experiments we found a robust upregulation of Na^+-independent L-[^{3}H]-glutamate uptake (blockable by unlabeled cystine) with inclusion of the xCT expression vector (Fig. 1). Glutamate is a well characterized substrate for system x_c^- transport activity (1, 15–18). The level of xCT expression, evaluated by L-[^{3}H]-glutamate uptake, was at least five- to tenfold above cells transfected with only control pBluescript vector (Fig. 1). Surprisingly, exogenous 4F2hc expression was not required for xCT-mediated system x_c^- activity. Moreover, the addition of 4F2hc expression vector significantly reduced cystine displaceable L-[^{3}H]-glutamate uptake (Fig. 1). These results indicate that system x_c^- activity is not limited by the availability of 4F2hc subunit in HEK293 cells. Inhibition of cystine transport caused by overexpression of 4F2hc may occur through altered trafficking of the xCT/4F2hc complex. Evidence from *Xenopus* oocytes suggests that 4F2hc is required for the maturation and cell surface localization of some transporters in the gpAAT family, perhaps by acting as a chaperone (21–24). By over-expressing 4F2hc, we may have been overloading and impeding the transport pathways used to deliver the xCT/4F2hc complex to the cell surface. Alternatively, increasing 4F2hc expression might alter the subunit stoichiometry with xCT in such a way that the transporter exhibits reduced activity or becomes non-functional. For example, alteration of subunit composition for ionotropic glutamate receptors or potassium channels leads to changes in their properties (25, 26).

It is also possible for excessive 4F2hc expression to saturate transcriptional and translational machinery that could otherwise be used for xCT production. To test this possibility, we performed experiments where a protein-producing construct, β-galactosidase, was substituted for 4F2hc, instead of pBluescript, which does not produce a protein product. The β-galactosidase gene expression was driven by a CMV promoter similar to that of xCT and 4F2hc. Using the β-galactosidase construct, we also observed a similar reduc-

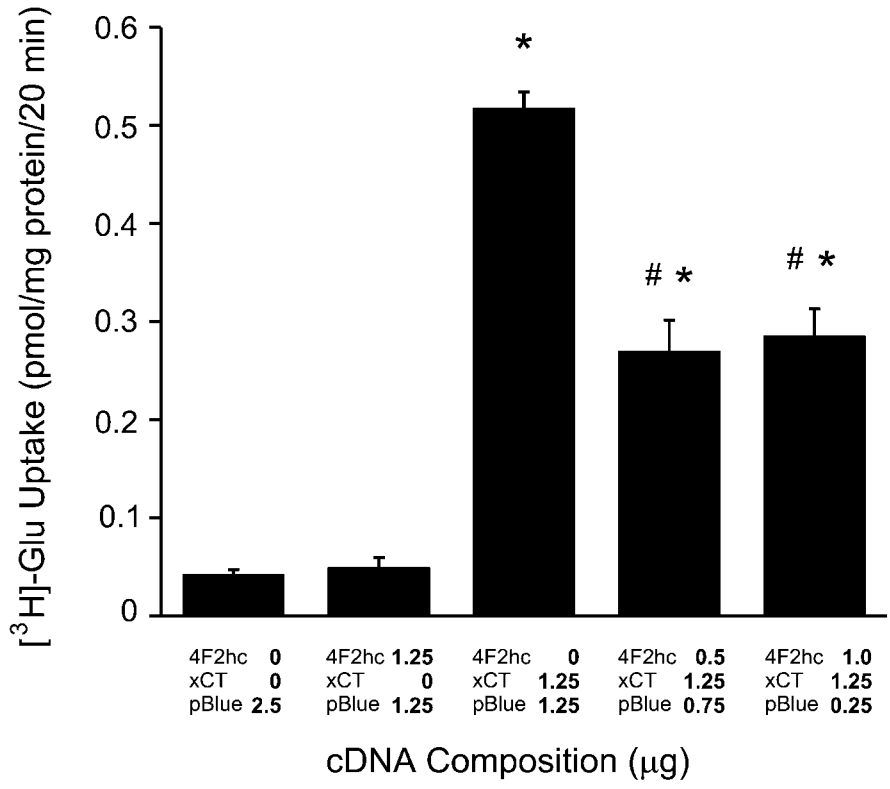

FIG. 1. L-[^{3}H]-glutamate uptake and the effect of varying levels of 4F2hc and xCT expression. Five groups of HEK293 cells were each transfected with the cDNAs indicated under each column and used to uptake 38 nM L-[^{3}H]-glutamate (42 Ci/mmol) as described under Materials and Methods. Two-tailed unpaired *t* tests were performed to evaluate the significance of differences between means. Columns 3, 4, and 5 were significantly different from control columns 1 and 2 (*), $P < 0.05$. Columns 4 and 5 were shown to be significantly different from column 3 (#), $P < 0.05$. The results are based on three separate experiments performed in duplicate. Nonspecific uptake (<5% of total) measured in the presence of 1 mM unlabeled L-cystine was subtracted from L-[^{3}H]-glutamate uptake in all columns.

tion in L-[^{3}H]-glutamate uptake upon inclusion of 4F2hc (data not shown), suggesting that saturation of transcriptional and translational machinery is likely not the primary cause for reduced transport activity.

Murine 4F2hc is ubiquitously expressed in many cell types (27). It is important as an antigen for lymphocyte activation and proliferation, and also plays a role in integrin activation (28–30). Only recently, has 4F2hc been shown to be an accessory protein for a family of amino acid transporters (2, 3, 31). In light of this evidence, we suspect that HEK293 cells express endogenous human 4F2hc at levels high enough to accommodate surplus xCT subunit. Although murine xCT has not been shown to functionally associate with human 4F2hc, there is evidence supporting that some murine and human homologues of gpAAT light chains and 4F2hc are interchangeable (6, 32). Quantitation of 4F2hc expression by Western blot with the well established murine 4F2hc monoclonal antibody will reveal the level of expression at which xCT-mediated transport becomes hindered (33). In future experiments, we plan to compare K_m and V_{max} values among cells transfected and untransfected with 4F2hc to determine whether the transporter function is altered at the cell surface or whether fewer transporters are reaching the cell surface due to impaired xCT trafficking.

To determine whether the blockade of xCT-mediated L-[^{3}H]-glutamate uptake by cystine occurred through a high affinity mechanism we examined the dose-inhibition relationship between unlabeled cystine and L-[^{3}H]-glutamate uptake. In this experiment a tracer level of L-[^{3}H]-glutamate (38 nM) was used for uptake in the presence of varying concentrations of unlabeled cystine (30–3000 μM). By fitting the inhibition data to a simple one-site inhibitor model [% control uptake = $100 - (100 \times I/(I + K_i))$] we calculated a K_i of (81 ± 13 μM) for unlabeled cystine. This K_i was from a single quadruplicate experiment using HEK293 cells transfected with 1.25 μg xCT and 1.25 μg 4F2hc. A similar value was obtained in a second separate experiment (data not shown). The K_i of unlabeled cystine for inhibiting xCT-mediated transport is consistent with Sato *et al.* who reported a K_m of 81 μM for cystine using *Xenopus* oocytes expressing xCT and 4F2hc cRNAs (1). However, the affinity for unlabeled cystine measured for endogenous system x_c^- activity is higher for N18-RE-105 cells (K_i = 23 μM) and immature cortical neurons (K_i = 31 μM) (15, 16). Since the experiments with

TABLE 1

Pharmacological Characterization of xCT-Mediated Transport

Compound	[^{3}H]-glutamate uptake (% control)
None	100
Cystine	1.8 ± 0.9*
Quisqualate	3.3 ± 1.3*
HCA	5.0 ± 0.6*
Glutamate	15 ± 3.1*
L-Aspartate	71 ± 3.7*
D-Aspartate	79 ± 8.2
Kainate	96 ± 5.0

Note. HEK293 cells were transfected with 1.25 μg xCT and 1.25 μg 4F2hc. All unlabeled compounds were used at 1 mM in the L-[^{3}H]-glutamate uptake assay protocol described under Materials and Methods. Control uptake is the uptake of (38 nM) L-[^{3}H]-glutamate (42 Ci/mmol) in the absence of any inhibitor (0.31 ± 0.04 pmol/mg protein/20 min). Values are the mean ± SEM of three separate experiments performed in quadruplicate. * Compounds that caused a significant inhibition of uptake, $P < 0.05$ (two-tailed paired t test).

endogenous system x_c^- were performed under conditions with Na^+ present (15, 16), the apparent higher cystine affinity could be attributed to both Na^+-dependent and independent cystine transport systems (34). Other possible determinants of substrate affinity that may account for this difference are subunit stoichiometry of the xCT/4F2hc complex and different subcellular microenvironments. Based of a simple Michaelis Menton model, we estimate a V_{max} of 1289 ± 148 pmol/mg protein/20 min for L-[^{3}H]-glutamate uptake, assuming a K_m of 160 μM as previously observed by Sato *et al.* (1). This value is comparable to the V_{max} measured for endogenous system x_c^- activity in N18-RE-105 cells and rat brain synaptosomes (15, 18).

The characteristics of xCT suggest that it is the core subunit of the system x_c^- cystine-glutamate exchanger. For example, Sato *et al.* have shown that xCT-mediated transport activity is Na^+-independent concurring with previous work on system system x_c^-, where low Na^+ medium did not significantly affect L-[^{35}S]-cystine uptake (1, 15). Another hallmark of this transport system found in brain and other cells such as fibroblasts or macrophages is a well-defined pharmacological profile. Using L-[^{3}H]-glutamate uptake assays in transfected HEK293 cells we have found a similar pharmacological profile when recombinant xCT-mediated transport and the endogenous brain system x_c^- transporter were compared (Table 1) (15–18). For example, compounds such as L-glutamate, L-homocysteate (HCA), and quisqualate all are effective blockers of L-cystine transport in neuronal cultures and transfected HEK293 cells (1, 12, 15–18). In contrast, similar compounds such as D/L-aspartate only have low affinity interactions with the transporter (1, 15, 16, 34). Other compounds such as kainate are completely ineffective at inhibiting transport (15, 16). Very recently, the expression and substrate specificity of human xCT/4F2hc was evaluated in HeLa cells (35). In this system, both cystine and glutamate were shown to be effective substrates. However, compounds relevant to oxidative stress-mediated toxicity such as quisqualate and HCA were not evaluated. This study found that xCT-mediated cystine transport was dependant upon the coexpression of endogenous 4F2hc (35), and transfection with xCT alone could not produce this activity. Perhaps the level of 4F2hc expression in the HeLa cell line is too low to accommodate the expression of excess xCT subunit.

To address the subcellular localization of xCT, we constructed a C-terminal xCT-GFP fusion protein. We then transfected the HEK293 cells with 1.25 μg xCT-GFP and 1.25 μg 4F2hc. Cells transfected with xCT-GFP exhibited a distinctly clustered pattern of fluorescence suggesting xCT localization within discrete subcellular compartments (Fig. 2). Cells transfected with a control non-xCT fused GFP (green lantern) showed a significantly more homogenous distribution of fluorescence. Fifty cells were observed from each group, where >90% of xCT-GFP had a clustered distribution of fluorescence and 100% of Green Lantern transfected cells had a homogenous distribution. Although xCT-GFP always formed clusters, its distribution did vary among the cells we observed. For example, some cells had large plasma membrane patches while others contained intracellular clusters and small plasma membrane puncta. We believe the transporter may reside near lysosomal and/or endosomal membranes contributing to the synthesis of glutathione (GSH) that is required for protein degradation, or may be situated near sites of cell–cell interaction (36). Perhaps, in the brain xCT is localized at sites of neuron–glial interaction where GSH is exported from glial cells for neuronal uptake (37). Subcellular fractionation and/or colocalization experiments using xCT antibodies and markers for subcellular compartments should reveal where xCT resides within the cell. It would also be interesting to determine whether enzymes involved in the synthesis of GSH, for example γ-glutamyl cysteine synthetase, colocalize with xCT (38). In the future, we plan establish the relationship between the localization of the transporter and its function. Unfortunately, the xCT-GFP construct appears to have little transport activity as assessed by L-[^{3}H]-glutamate uptake assays (data not shown). However, the targeting of xCT-GFP is likely to be unaffected since the N-terminal sequence is intact (C-terminal GFP fusion).

Data from our laboratory and others suggest that embryonic neuronal/glial cultures are dependent upon system x_c^- cystine transport for viability (9, 14–16, 39, 40). If transport is blocked by antagonists such as L-glutamate, HCA and quisqualate, neurons undergo cell death attributed to gradual GSH depletion (due to

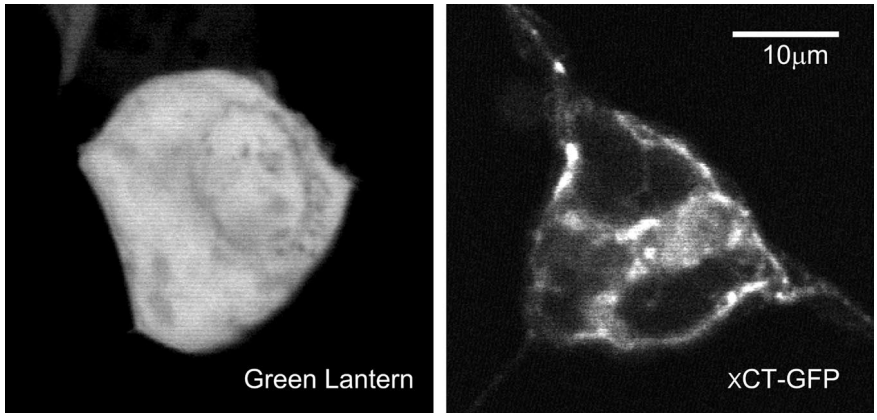

FIG. 2. Confocal images (single optical section with 1 μm Z resolution) showing xCT-GFP subcellular localization. HEK293 cells transfected with 1.25 μg xCT-GFP or 1.25 μg Green Lantern and 1.25 μg 4F2hc were allowed to express transfected proteins for ~72 h. Cells transfected with xCT-GFP showed a distinctly punctate distribution of fluorescence, while cells transfected with Green Lantern, a GFP without fused xCT, showed a more homogenous distribution. Fifty cells were observed from each group, where >90% of xCT-GFP had a clustered distribution of fluorescence and 100% of Green Lantern transfected cells had a homogenous distribution. This experiment was repeated with similar results.

lack of cystine uptake) and an accumulation of intracellular oxidants (9, 14–16, 39, 41). The fact that GSH levels are tightly associated with system x_c^- transport activity would suggest that control of xCT expression is a key event in maintaining intracellular GSH homeostasis. Interestingly, data from Ishii *et al.* suggests that xCT transport activity in macrophages is controlled through an antioxidant response element (ARE) mediated transcriptional mechanism (42, 43). Since this mechanism involves the ARE it can be induced by small molecule oxidants, such as tertiary butyl hydroquinone, dimethyl fumarate, and diethylmaleate, that affect the signal transduction machinery impinging on this sequence (44–47). These compounds have been found to protect against toxicity mediated by inhibition of cystine transport in N18-RE-105 cells. Therefore, our future goals are to both overexpress xCT transport activity and to use ARE inducers that enhance endogenous system x_c^- to bolster cellular GSH levels with the hope of protecting against oxidative stress associated with toxic conditions that may arise from ischemia, injury, neurodegenerative disease, or even trophic factor deprivation (41, 48).

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