Membrane Topology of System $x_c^-$ Light Subunit Reveals a Re-entrant Loop with Substrate-restricted Accessibility*

Received for publication, March 3, 2004, and in revised form, May 17, 2004
Published, JBC Papers in Press, May 19, 2004, DOI 10.1074/jbc.M402428200

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Heteromeric amino acid transporters are composed of a heavy and a light subunit linked by a disulfide bridge. 4F2hc/xCT elicits sodium-independent exchange of anionic L-cysteine and L-glutamate (system $x_c^-$). Based on the accessibility of single cysteines to 3-(N-maleimidylpropionyl)/biocytin, we propose a topological model for xCT of 12 transmembrane domains with the N and C termini located inside the cell. This location of N and C termini was confirmed by immunofluorescence. Studies of biotinylation and accessibility to sulfhydryl reagents revealed a re-entrant loop within intracellular loops 2 and 3. Residues His$^{110}$ and Thr$^{112}$, facing outside, are located at the apex of the re-entrant loop. Biotinylation of H110C was blocked by xCT substrates, by the non-permeable reagent (2-sulfonatoethyl)-4-carboxyphenylglycine, and of H110C was protected by L-glutamate and L-cysteine with an IC$_{50}$ similar to the $K_m$. Protection was temperature-independent. The data indicate that His$^{110}$ may lie close to the substrate binding/permeation pathway of xCT. The membrane topology of xCT could serve as a model for other light subunits of heteromeric amino acid transporters.

Heteromeric amino acid transporters (HATs)$^1$ are composed of a heavy subunit and a light subunit (LSHAT) linked by a conserved disulfide bridge (1, 2). The heavy subunit is important for trafficking of the heterodimer to the plasma membrane, whereas the light subunit confers transport function and specificity. Two heavy subunits are known, 4F2hc and rBAT, which combine with a range of LSHATs to form several transport systems. 4F2hc heterodimerizes with xCT to elicit sodium-independent transport of anionic L-cysteine and L-glutamate by a 1:1 obligatory exchange (system $x_c^-$). xCT is expressed in most cell lines, in activated macrophages and in the brain (4, 5). System $x_c^-$ functions physiologically for cysteine uptake and glutamate efflux because of the low and high intracellular concentrations of cysteine and glutamate, respectively. Cytosolic concentrations of cysteine are kept low because of its rapid reduction to cysteine, the rate-limiting substrate for the synthesis of intracellular glutathione (4, 6). Consistent with a role of xCT in cellular antioxidant defense, the xCT gene carries an electrophil response element that may mediate the up-regulation of system $x_c^-$ activity by oxygen in macrophages and fibroblasts (5, 7). Moreover, system $x_c^-$ in macrophages and microglia is envisaged as a mechanism for glutamate efflux potentially leading to excitotoxic neural injury in pathological states (5, 8, 9).

Little is known of the protein structure or the structure-function relationships of HATs. rBAT and 4F2hc are believed to be type II membrane glycoproteins with an intracellular N terminus, a single transmembrane domain, and a bulky (N-glycosylated) extracellular domain (1). The N terminus of 4F2hc has a cytoplasmic location (10). In contrast, LSHATs are not N-glycosylated and show a hydrophobicity profile suggesting 12 transmembrane domains. Reconstitution studies with the b$^{5-}$-AT light subunit showed that the rBAT heavy subunit is not necessary for the basic transport function (11). Then the relevant functional determinants should lie on the LSHATs. Our knowledge of the structure-function relationships of LSHATs rely on a few single residues. A naturally occurring interspecific change (W234L) slightly modified the $K_m$ of LAT1 (12). Among the mutations identified in y$^{-}$/LAT1 causing lysinuric protein intolerance (13) and those in b$^{5-}$-AT causing cystinuria (14), L334R (y$^{-}$/LAT1) and A354T (b$^{5-}$/AT) inactivate the transporter (11, 15). Finally, there is evidence that Cys$_{327}$ lies close to the substrate binding site/permeation pathway of xCT (16).

To describe the membrane topology of LSHATs, we performed cysteine scanning accessibility studies using xCT as a model. Our results are compatible with 12 transmembrane domains and with intracellular N and C termini. Moreover, evidence is presented in support of a re-entrant loop between transmembrane domains 2 and 3, the accessibility of which is restricted by xCT substrates/inhibitor.
Construction of the Cysteineless xCT and the Single Cysteine Mutants—The seven native cysteine residues of the wild-type transporter (residues 157, 158, 159, 271, 327, 414, and 435) were each replaced by serine (Cys-less xCT) by sequential site-directed mutagenesis (QuikChange™, Stratagene), using human xCT in pNKS2 as a template. The mutated regions were excised by digestion and subcloned back into the original plasmid. The Cys-less xCT was then subcloned by enzyme digestion into the pCDNA4 HisMax vector to insert a six-histidine tag at its N terminus (His-Cys-less). Single cysteine mutants were made in the same way by using His-Cys-less as a template. The mutations were confirmed by DNA sequencing with a d-Rhodamine dye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer Life Sciences) in an Abi Prism 377 DNA Sequencer.

Cell Culture and Transfections in HeLa Cells—HeLa cells were cultured as described (11) and grown at 37 or 33 °C, as indicated. Transient transfections were performed by standard calcium phosphate precipitation as described (15) in 10-cm plates with a mixture of DNA containing 2 μg of pEGFP (Clontech) and 18 μg of the indicated pCDNA4 HisMax xCT construct. The green fluorescence protein-encoding plasmid was included to monitor transfection efficiencies, which ranged from 70 to 90% as assessed by fluorescence-activated cell sorter analysis for BM labeling and transport assay experiments.

Transport Measurements—Transient transfected HeLa cells were cultured in 24-well plates. 48 h later, influx rates of 25 μM L-[35S]cysteine (American Biosciences) and 50 μM L-[3H]glutamate (American Radiolabeled Chemicals) were measured for 45 s (linear conditions) as described previously (15). Induced transport values were calculated by subtracting transport in mock transfected cells (i.e. empty pCDNA3-transfected cells). Each independent experiment was performed with 4–6 replicates. Efflux measurements were performed as described in the legend to Supplemental Fig. A. Nonradioactive amino acids and chemicals were purchased from Sigma.

Labeling with BM and Purification on Ni-NTA Beads—Labeling with 500 μM BM was carried out as described previously (17), with the following modifications. After the biotinylation step, the cells were lysed with 100 μL/well of a solution containing 0.3 M NaCl, 1% Triton X-100, 0.025 M imidazole, protease inhibitor mixture (phenylmethanesulfonyl fluoride, aprotenin, leupeptin, and pepstatin), and 0.05 M NaPO4 (pH 7.4). The cells were scraped, transferred into a 1.5-mL tube, and incubated on a rotating orbital. After 30 min, the insoluble material was removed by centrifugation at 12,000 rpm for 10 min. The supernatants were incubated with 20 μL of Ni-NTA-agarose beads (Qiagen) pre-equilibrated in 150 μL of lysis solution for 30 min at 4 °C. After 1.5 h of incubation at 4 °C with the solubilized cellular proteins, the beads were washed twice in 750 μL of a PBS solution containing 0.5 M NaCl and 0.05 M imidazole (pH 7.4). The proteins were eluted with 45 μL of elution buffer (PBS solution containing 0.5 M NaCl and 0.05 M imidazole, pH 7.4) after 15 min of shaking incubation at room temperature. When indicated, the cells were permeabilized with streptolysin O (SLO) immediately before the biotinylation step, as described (17). The SLO buffer, as recommended by the supplier, was 10 mM phosphate buffer (pH 7.4) supplemented with 1 mM dithiothreitol. 4-fold concentrated SDS-PAGE loading buffer and 100 mM dithiothreitol were added to the eluates from the Ni-NTA beads. After SDS-PAGE, the eluates were transferred onto nitrocellulose, and the biotinylated proteins were detected by using streptavidin-peroxidase (Roche Applied Science) and ECL (Amerham Biosciences) according to the instructions of the manufacturer. For each of the mutants, biotinylation was performed at least three times. Densitometry was carried out with the program Gene Tools from Syngene.

Effect of MTS Reagents on Transport—HeLa cells were transfected with the indicated plasmid and cultured in 24-well plates. 48 h later the cells were incubated for 5 min at room temperature with uptake solution without amino acid (157 mM methyl glucamine, 2.8 mM CaCl2, 1.2 mM MgSO4, 5.4 mM KCl, 10 mM HEPES, pH 7.5) containing the indicated concentrations of MTSET, MTSEA, or MTSES. For protection assays, unlabeled l-cysteine, L-glutamate, or l-arginine was added to 10 mM MTSES solution. Then cells were washed three times with uptake solution (without amino acid) and subsequently assayed for transport activity. For substrate-protection dependence curves, MTSES was used at 1 mM for 10 min of incubation time in the presence of a range of l-glutamate or l-cysteine concentrations. MTSES reagents (Toronto Research Chemicals) were dissolved as a 1 mM stock solution in Me2SO. After dilution in the medium to the final concentration, the reagents were used immediately.

Immunofluorescence Microscopy—HeLa cells were transfected on cover slides (Marienfeld) with the indicated plasmid and 2 μg of pEGFP (Clontech). After 48 h, the cells were fixed with 3% paraformaldehyde in PBS solution and permeabilized for 10 min with PBS with 0.1% Triton X-100. After 30 min of incubation with 1% bovine serum albumin, each cover slide was incubated for 1 h at room temperature with anti-Xpress antibody (Invitrogen), diluted 1:100 in PBS containing 20 μg/ml glycine and 1% bovine serum albumin. The slides were washed twice in PBS-glycine and incubated for 45 min at room temperature with Texas Red-conjugated goat anti-mouse (Molecular Probes), diluted 1:100. After two washes in PBS, the slides were mounted on microscope slides (Menzel-Glaser) with mowiol. The images were obtained from the Leica TCS NT confocal microscope, at the Scientific Services of the Barcelona Science Park (Fig. 1). Transfection efficiencies in these experiments (i.e. green fluorescence protein signal) ranged from 50 to 70%.

Western Blot Analysis of His-tagged xCT—Eluates from the purification on Ni-NTA beads containing 4-fold concentrated SDS-PAGE loading buffer and 100 mM dithiothreitol were loaded for SDS-PAGE (10% polyacrylamide) and transferred onto Immobilon (Millipore Iberica). The membranes were blocked with 3% bovine serum albumin in Tris-buffered saline (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) for 1 h at 37 °C. Anti-Xpress antibody (Invitrogen) was used at a 1:5000 dilution in 1% bovine serum albumin and 0.05% Tween 20 in Tris-buffered saline, according to the manufacturer’s instructions. Horseradish peroxidase-conjugated goat anti-mouse antibody (Sigma) was used as a secondary antibody at 1:50,000 dilution. Antibody bonding was detected using ECL Western blot detection system (Amerham Biosciences).

Oocyte Dixon Plot and Efflux Studies—Oocyte origin, management, cRNA synthesis, and injections were as described elsewhere (18). Dixon plot of 4-S-CPG inhibition of transport and efflux determination via 4F2hc/xCT were performed as described in the legends to Supplemental Figs. C and D.

Kinetic Data Analysis—Nonlinear regression fits of experimental and calculated data to estimate Vmax and Km (Michaelis-Menten equation), half-life times (t1/2) for transporter inactivation, IC50 values for substrate protection, and K for 4-S-CPG were performed with GraphPad Prism as described (16).

RESULTS

Localization of the N and C Termini of xCT—Two tagged versions of xCT were constructed, His-xCT (i.e. His-Xpress at the N terminus) and xCT-myc (i.e. myc at the C terminus). Transfection of human His-xCT alone or in combination with human 4F2hc resulted in the induction of both 50 μM L-[3H]glutamate (4-fold) and 25 μM L-[35S]cysteine (6-fold) uptake over background (Supplemental Fig. A). The His-xCT-induced transport was similar to that elicited by nontagged xCT (88 ± 9 and 84 ± 7% of the transport of 25 μM L-[35S]cysteine and 50 μM L-[3H]glutamate in nontagged xCT-transfected cells, respectively; data not shown; n = 5 independent experiments). The characteristics of the His-xCT-induced transport agree with...
those obtained previously with wild-type xCT and 4F2hc (3, 4, 16): sodium independence (data not shown), $K_m$ values for substrates in the $\mu M$ range (Table I), and L-cysteine trans-stimulation of L-glutamate efflux (Supplemental Fig. A). Similarly, xCT-myc also elicited system xc\texttext{-}^-\textsuperscript{-} transport activity ($4\times$ fold of 25 $\mu M$ L-[^35]S]cysteine over background, representing $67\%$ of the untagged transporter; $n = 8$). Tag immunodetection of His-xCT and xCT-myc occurred only when the cells

**Table I**

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<th>Glutamate</th>
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<td>$V_{max}$ (pmol/min/mg protein)</td>
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<td>224 $\pm$ 78 (4)</td>
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<td>His-H110C</td>
<td>192 $\pm$ 53 (4)</td>
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<td>8121 $\pm$ 599</td>
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<td>His-H110K</td>
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<tr>
<td>His-Cys-less</td>
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<td>His-H110C-Cys-less</td>
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**Fig. 2.** Transport activity of His-tagged single cysteine mutants of xCT. The indicated native residues were replaced by cysteine. L-[^35]S]Cysteine transport was measured in HeLa cells expressing these mutants or the parental His-tagged cysteine-less xCT (His-Cys-less). The values are expressed as percentages relative to the His-Cys-less. Each bar is the mean $\pm$ S.E. ($n = 3$–8). His-Cys-less-induced transport was 1051 $\pm$ 109 pmol/min/mg protein ($n = 12$).
were permeabilized (Fig. 1). In this case, in addition to intracellular signal, a clear label of the plasma membrane was obtained. This strongly supports the intracellular location of both ends of xCT.

HeLa cells express 4F2hc and are devoid of rBAT expression (11). This suggests that endogenous 4F2hc drives transfected xCT to the plasma membrane. This is in agreement with previous results obtained in HeLa cells for asc-12 and xCT (Cys\textsuperscript{trans}) is shown. B, single cysteine residues expected to be located outside were pretreated or not with 1 mM MTSET and then biotinylated with BM. C, single cysteine residues expected to be located within the cell were biotinylated with BM. When indicated, biotinylation (BM) was performed after streptolysin O permeabilization (SLO) or after N-ethylmaleimide pretreatment and consequent SLO permeabilization (N+S). The positions of two markers are indicated at the left (B and C). Biotinylation was performed three to five times with similar results.

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Membrane Topology of xCT, a cysteine accessibility strategy was performed (17). An N-terminal His-tagged cysteineless xCT (His-Cys-less) was obtained by mutating the 7 endogenous cysteine residues to serine. His-Cys-less showed transport characteristics similar to those of wild-type xCT. In cells grown at 33 °C to increase transport induction, as learned with rBAT/b\textsubscript{0},/H\textsubscript{11001}/H\textsubscript{9262}/H\textsubscript{51001}/H\textsubscript{35001}/H\textsubscript{13001}/H\textsubscript{30001}, xCT-induced transport was 3065 ± 142 pmol/mg protein min in His-xCT-transfected cells and 1045 ± 75 pmol/mg protein min in His-Cys-less-transfected cells (i.e. 34% ± 5% of His-xCT) (n = 8). This represents 7.6- and 3.3-fold transport activity over background conditions (462 ± 50 pmol/mg protein min in mock-transfected cells; n = 8) in His-xCT- and His-Cys-less-transfected cells respectively. His-Cys-less showed similar apparent $K_m$ for cysteine and 3.6-fold higher apparent $K_m$ for glutamate than His-xCT (Table I). Mutation of cysteine residue 327 to
serine is responsible for the increased glutamate $K_m$ (16). Therefore, we have used His-Cys-less as the basis for the topology determination. Fig. 2 shows the positions where single cysteine residues were introduced in His-Cys-less and the transport activity of L-[35S]cysteine induced upon transfection in HeLa cells. Single cysteine mutants with transport activities below 25% of His-Cys-less were not considered for further studies.

A typical experiment, in which biotinylated xCT derivatives are visualized, is shown in Fig. 3A. HeLa cells expressing the xCT constructs were reacted with BM. After solubilization of the membrane proteins, the His-tagged transporters were purified on Ni-NTA beads. The covalently bound biotin was readily visualized after SDS-PAGE in reducing conditions by probing the blotted proteins with streptavidin-peroxidase. The biotinylated His-tagged xCT (His-xCT) ran as a band around 40 kDa, which is consistent with the mobility of immunodetected His-xCT (Fig. 3B). Other labeled bands are not specific because they were detected in nontagged xCT-transfected cells (data not shown) and in His-Cys-less. Precinubcation of the cells expressing His-xCT with the membrane-impermeable sulfhydryl reagent MTSET (21) blocked biotinylation only partially. This suggests that some of the seven endogenous cysteines are accessible from the external medium (i.e., MTSET-sensitive label), whereas others are accessible to the slightly permeable BM, suggesting access to the internal milieu. As shown in Fig. 3B, overexpressed His-xCT showed another faint band of ~80 kDa, representing most probably a homodimer. This dimerization in reducing SDS-PAGE has been previously reported for other LSHATs (e.g., b0,+AT; Refs. 14 and 22). Biotinylation of the ~80-kDa band was not detected, most probably because of the low expression of this band and the presence of nonspecific biotinylated bands in this mobility range.

When Leu163 was replaced by cysteine (in His-Cys-less background), biotinylation was again apparent as a specific ~40-kDa band. Precinubcation with MTSET completely blocked this biotinylation. This suggests that the cysteine introduced at the 163 position is accessible from the external medium. In contrast, cysteine residues introduced at positions 187 (S118C) or 260 (E260C) did not result in biotinylation of the 40-kDa band. This lack of biotinylation seems not to be due to a loss of expression of the mutants as they showed significant transport activity (Fig. 2). When SO was used to permeabilize HeLa cells expressing E260C, the transporter became biotinylated (Fig. 3A). In this case, precinubcation with the highly membrane permeable N-ethylmaleimide blocked this biotinylation. As expected, SLO permeabilization did not increase biotinylation of the externally located residue 163 (E163C) (data not shown). This suggests that the cysteine introduced at the 260 position is accessible from the inside. In contrast, S118C was not biotinylated after SLO (Fig. 3A). This negative result is ambiguous; it may mean that this residue is located in hydrophobic surroundings within the membrane, is facing an aqueous access channel too narrow to be accessible to the bulky BM, or is hidden within a particular secondary structure of the xCT transporter or by interacting proteins (e.g., the heavy subunit 4P2hc). Biotinylation of two bands around ~80 kDa (Fig. 3A) is unspecific because biotinylation of these bands are also visible in nontagged xCT- and His-Cys-less-transfected cells (data not shown).

Biotinylation Screening to Establish the Membrane Topology of xCT Subunit—To identify the membrane topology of additional sites, we introduced single cysteines at many other positions of His-Cys-less. Fig. 4A shows a 12-transmembrane domain model of xCT based on the HMMTOP algorithm (23). Residues with an expected extracellular location at positions Asn72 (EL1–2), Leu163 (already shown in Fig. 3A), Gln219 (EL5–6), Ala249 (EL7–8), Ser287 (EL9–10), and Ser495 (EL11–12) when mutated to cysteine showed biotinylation, which was blocked by MTSET (Fig. 4B). This showed that these residues are accessible from the external medium, as expected. Fig. 4C shows biotinylation of residues expected to be located inside (Fig. 4A). Positions Ser11 (N terminus), Thr102 (IL2–3), Ser181 and Phe183 (IL4–5), Glu260 (IL6–7; also shown in Fig. 3A), Glu353 and His365 (IL8–9), His418 (IL10–11), and Asp499 (C terminus) when mutated to cysteine become strongly biotinylated only after permeabilization with SLO. Moreover, N-ethylmaleimide blocked biotinylation of these residues. Several residues (S11C, T102C, E260C, H363C, H418C, and D499C) showed slight biotinylation without permeabilization. This is most probably due to the fact that BM has some permeability through the plasma membrane (17). Indeed, this biotinylation was MTSET-insensitive (data not shown), and biotinylation increased strongly after permeabilization with SLO. This shows that all these residues are accessible from inside. Biotinylation of residues within putative IL4–5 was difficult. Thus, in addition to the intracellularly accessible residues Ser181 and Phe183, other residues within this loop (Ile185, Ser185, Ser187 (Fig. 3A), Ala188, Ile190, and Ile192) showed no biotinylation even after permeabilization (data not shown). The inside location of the N and C terminus suggested by the biotinylation.
studies confirm the immunofluorescence results shown in Fig. 1. Finally, residues predicted to be located within transmembrane domains (Lys105 in transmembrane domain 4, Cys897 in transmembrane domain 5, and Cys8127 and Ala337 in transmembrane domain 8) showed no biotinylation even after permeabilization with SLO (data not shown). In all, these results give experimental support to the 12-transmembrane domain model of xCT.

**Biotinylation of Single Cysteine Residues within IL2–3—**

Mutant His110C showed biotinylation from the external medium (biotinylation is blocked by the membrane impermeable MTSES; Fig. 5), whereas the neighboring residue Thr122 (mutant T102C) showed biotinylation from inside (Fig. 4C). This fostered more extensive screening of the intracellular loop 2–3 (Fig. 4C). Residues Thr125 and Glu126, flanking position His110 at 6 or 8 residues, showed biotinylation from inside. This suggests a re-entrant loop-like structure within IL2–3. One of the closest His110-flanking positions, G109C showed biotinylation only after permeabilization with SLO (Fig. 4C). The other closest flanking position, Tyr111, is a fully conserved residue (Supplemental Fig. E), which when mutated to cysteine resulted in very little transport activity (Fig. 2) and mistrafficking to the plasma membrane (Supplemental Fig. B). Other positions within IL2–3 gave no accessibility information. Thus, positions Lys105, Thr112, Phe118, Leu121, and Pro122 (data not shown) when mutated to cysteine showed no biotinylation even after permeabilization (Fig. 4C). Positions Ala123, Phe124, Arg126, and Glu130 were not analyzed because of the very low residual transport activity of the corresponding cysteine mutants (Fig. 2).

The biotinylation of His-H110C-Cys-less from outside prompted studies with membrane-impermeable cysteine reagents. Preincubation with the negatively charged MTSES blocked biotinylation, whereas the positively charged MTSET did not (Fig. 5). This supports the external accessibility of residue His110 and suggests that negatively charged reagents may have greater accessibility to this residue from outside than positively charged reagents. System xc\(^{-}\) exchanges anionic substrates (glutamate and anionic cysteine) (3, 4). This coincidence in negative charge fostered substrate protection experiments of H110C biotinylation. L-Glutamate and L-cysteine, but not L-arginine (neither a substrate nor an inhibitor of xCT), blocked biotinylation of H110C (Fig. 5). Recently it has been shown that 4-S-CPG is a nonsubstrate competitive inhibitor of system xc\(^{-}\) (Supplemental Fig. D). Interestingly, a saturating concentration of 4-S-CPG (1 mM) blocks H110C biotinylation to a similar extent to a saturating concentration of L-glutamate (10 mM) (Fig. 5). This suggests that occupation of the substrate binding site prevents accessibility of residue His110 from outside.

**Effect of Methanethiosulfonate Derivative Reagents on Single Cysteine Residues within IL2–3—**

Next, we investigated transport inactivation by MTS reagents of single cysteine residue mutants of IL2–3. To compensate the differential reactivity of the MTS reagents used with free cysteine in solution (MTSES is 10 and 4 times less reactive than MTSET and MTSEA, respectively (21)), MTSES was used at 10 mM, MTSEA was used at 10 mM, and MTSET was used at 2.5 mM, and MTSET was used at 1 mM (Fig. 6). As expected, His-Cys-less transport activity was unaffected by the MTS reagents. Interestingly, only His-H110C-Cys-less was inactivated by MTSES and to a lesser extent by the membrane permeable MTSEA. In contrast, MTSET did not inactivate His-H110C-Cys-less-induced transport. This order of inactivation was also obtained when the three MTS reagents were used at 1 mM for 10 min. Residual transport activity was 32.6 ± 9.3, 58.7 ± 3.2, and 107.0 ± 8.0% after MTSES, MTSEA, and MTSET treatment, respectively (\(p < 0.05\) between the three groups; Student \(t\) test; means ± S.E.; representative experiment with five replicas). This confirms the previous biotinylation studies and strongly supports the outside accessibility of residue His110. MTSES did not abolish completely His-H110C-Cys-less-induced transport. Thus, at maximal conditions of inactivation (10 mM MTSES for 5 min; Fig. 7), similar residual transport activity was obtained at 25 \(\mu\)M (17.4 ± 2.9%; \(n = 8\)) and 350 \(\mu\)M (16.7 ± 5.8%; \(n = 3\)) L-[\(^{35}\)S]cysteine (i.e. four times below and three times above apparent \(K_m\), respectively; Table I). This indicates that MTSES reduces ∼6-fold the transport activity of His-H110C-Cys-less, probably affecting \(V_{\text{max}}\) treatment with the impermeable MTSES and MTSET and the permeable MTSEA resulted in the stimulation of His-T112C-Cys-less transport activity (up to 80% increase) (Fig. 6). This suggests that Thr112 is accessible from the outside. The permeable MTSEA did not affect the transport activity of T102C, G109C, or E116C (Fig. 6), which are biotinylated from the inside (Fig. 4). These residues might be modified by MTSEA, but transport activity was not affected. Indeed, MTSEA did block biotinylation of G109C and E116C (data not shown), showing the accessibility of MTSEA to these residues. In contrast, MTSEA did not block biotinylation of T102C, suggesting that this residue is not accessible to the reagent. In all, biotinylation and MTS reagent treatment studies suggest that the accessibility within this re-entrant loop is restricted to posi-

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**Fig. 6.** Effect of MTS reagents on single cysteine mutants in IL2–3.

HeLa cells expressing His-tagged cysteineless xCT (His-Cys-less) and the indicated mutants, in the same cysteineless background, were left untreated (none) or treated with 10 mM MTSES, 2.5 mM MTSEA, or 1 mM MTSET for 5 min. Then the cells were washed and assayed for 25 \(\mu\)M L-[\(^{35}\)S]cysteine transport. The data (means ± S.E.; \(n = 2–4\)) represent percentages relative to His-Cys-less-induced transport without treatment. Inactivation of His-H110C-Cys-less by MTSES and MTSEA was statistically significant \((p < 0.001;\) Student \(t\) test). This MTSES inactivation was greater than that by MTSEA \((p < 0.05;\) Student \(t\) test). Stimulation of His-T112C-Cys-less activity by MTSES, MTSEA, and MTSET was statistically significant \((p < 0.001;\) Student \(t\) test).
alone or in combination with 10 mM L-arginine (induced transport. The cells were pretreated or not with MTSES for 30 s ent amino acids (aa) from MTSES inactivation of His-H110C-Cys-less-
B each of the representative experiments shown). Fraction occurred at substrate concentrations (Fig. 8) similar to the
an inhibitor, did not protect. Moreover, half-maximal protec-
tion was by 10 mM MTSES for 10 min at 25 °C, alone or in combination with different concentrations of L-cysteine (10, 25, 50, 75, 150, 250, and 350 μM) (upper graph) or L-glutamate (50 μM, 100 μM, 250 μM, 500 μM, 750 μM, 1 mM, 2 mM, 5 mM, and 10 mM) (lower graph). Then transport of 25 μM L-[35S]cysteine was measured. These conditions of inactivation by MTSES were chosen because maximal inactivation was accomplished (88% ± 7%) with a low inactivation rate (t1/2 = 285 ± 114 s; representative experiment with five replicas) (data not shown). In this instance, residual transport activity is a good estimation of the rate of inactivation. The data represent the percentages of protection respect to the transport elicited without MTSES treatment (means ± S.E. from a representative experiment).

Fig. 7. Substrate protection from H110C-dependent MTSES-mediated transport inactivation. HeLa cells expressing H110C in a cysteineless background (His-H110C-Cys-less) were pretreated with 10 mM MTSES and then assayed for 25 μM L-[35S]cysteine transport. A, time-dependent inactivation of His-H110C-Cys-less-induced transport after MTSES preincubation at 25 °C or 4 °C, alone or in the presence of 10 mM L-glutamate (Glu). The data (means ± S.E.) are expressed as the fractional remaining activity. His-Cys-less-induced transport in untreated cells for the protection experiments at 25 and 4 °C was 769 ± 105 and 1118 ± 152 pmol/min/mg protein, respectively (five replicas in each of the representative experiments shown). B, protection by different amino acids (aa) from MTSES inactivation of His-H110C-Cys-less-induced transport. The cells were pretreated or not with MTSES for 30 s alone or in combination with 10 mM L-arginine (Arg), 10 mM L-glutamate (Glu), or 350 μM L-cysteine (Cys/C). Transport is expressed in pmol/min/mg protein (means ± S.E. from a representative experiment).

Substrate Protection of MTSES Inactivation of His-H110C-Cys-less—The blockade by glutamate, 4-S-CPG, and MTSES of His-H110C-Cys-less biotinylation strongly suggests that substrates would delay the reaction of MTSES inactivation, as it is shown in Fig. 7. Preincubation with L-cysteine and L-glutamate strongly protected against inactivation by MTSES at 25 °C. Indeed, the t1/2 calculated for 10 mM MTSES were 16 ± 3 s in the absence of amino acid and 394 ± 83 s in the presence of glutamate (data from a representative experiments done with four replicas). As expected, L-arginine, neither a substrate nor an inhibitor, did not protect. Moreover, half-maximal protection occurred at substrate concentrations (Fig. 8) similar to the

for His-H110C-Cys-less (Table I); The IC50 values for L-glutamate and L-cysteine were 1078 ± 223 and 807 ± 302 μM (in two independent experiments) and 97 ± 30 μM (representative experiment), respectively. This suggests that substrate protection of the Cys110 residue occurs at a step within the transport process.

At least two mechanisms may account for protection: substrates may directly compete with MTSES for the substrate binding site/permeation pathway, or alternatively a substrate-induced conformational change may prevent the access of MTSES to Cys110. It is generally accepted that large conformational changes in proteins are much more sensitive to temperature changes than substrate binding (25, 26). This feature has been exploited experimentally to detect conformational changes in various carriers, such as the serotonin transporter (27), the neuronal glycine transporter (28), and the xCT transporter (16). Indeed, xCT-induced transport is completely abolished at 4 °C (data not shown), even though the temperature dependence of xCT substrate binding is unknown. We examined the time course of the modification of His-H110C-Cys-less by 10 mM MTSES in the presence of 10 mM glutamate at 4 °C (Fig. 7A). MTSES, as expected, inhibited uptake more slowly than at 25 °C (t1/2 = 51 ± 20 s) (representative experiment). L-Glutamate also protected from the inactivation at 4 °C (t1/2 = 339 ± 121 s) (representative experiment). Thus, L-glutamate protects the Cys110 residue at a step in the transport cycle most likely prior to temperature-sensitive substrate-induced conformational changes. The degree of protection by glutamate was lower at 4 °C (t1/2 increased 7-fold) than at 25 °C (t1/2 increased 24-fold). This can be explained by additional tempera-
**Fig. 9.** Topological model of human xCT. The 12 transmembrane domains are numbered, and the re-entrant loop is marked (I). The dark circles indicate biotinylation from outside, and the stars indicate biotinylation from inside. The large white circles indicate no biotinylation even after SLO permeabilization, and the crossed circles indicate very low activity when substituted by cysteine (<25% of His-Cys-less xCT). The black circle within the open circle (Thr112) indicates the accessibility from the outside to MTSES and MTSET but not to BM. Cys158 involved in the disulfide bridge with 4F2hc is indicated with a C in a square.

Discussion

To our knowledge, this is the first experimental evidence for the overall membrane topology of transporter xCT as a model for the LSHATs. Our data (Figs. 3 and 4) revealed residues of the xCT transporter compatible with 12 transmembrane domains and the N and C termini located intracellularly (Fig. 9). This is in agreement with predictions by membrane topology algorithms (e.g. HMMTOP; Fig. 4A). Independent evidence for the intracellular location of the N and C termini was obtained by immunodetection of N- and C-tagged versions of xCT only after plasma membrane permeabilization (Fig. 1). The intracellular location of the C terminus of LSHATs is in agreement with functional concatenamers Nterm-b0, T-rBAT-Cterm (29) and Nterm-xCT-4F2hc-Cterm. The N terminus of 4F2hc is located intracellularly (10), and therefore these concatenamers would only be functional with light subunits with an intracellular C terminus. Similarly, the extracellular location of residue Leu163 (extracellular loop EL3) (Figs. 3A and 4B) is in agreement with the participation of the conserved neighbor residue Cys158 in the disulfide bridge that covalently links the light and heavy subunits of HATs (3, 30). The 12-transmembrane domain model of xCT gives structural support to the transport function of LSHATs in the absence of the heavy subunit, as has been demonstrated for b5/AT (11).

Biotinylation of residues after permeabilization with SLO within the putative IL4–5 of xCT was difficult. IL4–5 comprises residues 180–193 (Fig. 9). Biotinylation was only accomplished at the ends of this loop (S181C and F193C). Cysteine substitution mutants of residues Ile182, Ser185, Ser187, Ala188, Ile186, and Ile188 showed no biotinylation after SLO permeabilization (Fig. 9). Similarly, no topology information is available on the C-terminal half of the IL2–3. Residues Phe122, Leu121 (Fig. 4C), and Pro122 (data not shown) showed neither biotinylation nor inactivation with MTS reagents, and residues Ala123, Phe124, Arg126 and Glu130 do not tolerate replacement by cysteine (Table 1). This suggests that most of IL4–5 and the C-terminal half of IL2–3 is hidden. Whether this is due to insertion in the plasma membrane or interaction within these intracellular loops or with the intracellular N terminus of 4F2hc is unknown at present. Further studies are needed to test these possibilities.

Biotinylation of residues within the N-terminal half of the putative IL2–3 suggests a re-entrant loop structure (Fig. 9). Residues Thr112, Gly109, and Glu116 are biotinylated from inside, whereas residue His110 is biotinylated from outside (Figs. 4c and 5). Moreover, mutants H110C and T112C in a cysteineless background, are inactivated or stimulated by the membrane-impermeable reagent MTSES, respectively (Fig. 6). Thus, there are two residues (His110 and Thr112) with accessibility from outside flanked by residues with accessibility from inside (Thr112, Gly109, and Glu116) within a stretch of 15 residues (Fig. 9). This arrangement is reminiscent of pore loops from ion channels (31, 32), glutamate receptors (33) and more recently described transporters (e.g. glutamate transporters GLT1, EAAT1, and GltF; the Na+/Ca2+ exchanger NCX1; and the citrate/malate transporter CimH (17, 34–39). The re-entrant loop of xCT shares common characteristics with those mentioned above: 1) Substrates (L-glutamate and L-cysteine) and the nontransportable inhibitor (4-S-CPG) protect against the modification of the key residue H110C (Figs. 5, 7, and 8). 2) The re-entrant loop has a restricted external accessibility, apparently to residues His110 and Thr112. Topology of Tyr111 could not be determined with the methodology available because of a plasma membrane trafficking defect of His-Y111C-Cys-less (Supplemental Fig. B). His110 is, among the residues studied within IL2–3, the only one with BM accessibility from outside. (Figs. 4C and 5). This suggests that this residue is located within the apex of the re-entrant loop. 3) As indicated by the solved structure of potassium channels, it appears that the apex of re-entrant pore loops does not reach the levels of the phospholipids head groups on the other side of the membrane (31). BM reacts only with cysteines exposed to the aqueous phase. The clear biotinylation of H110C from outside suggests few steric restrictions within the external access channel to the apex of the xCT re-entrant loop. Residue G109C, next to the external apex of the re-entrant loop, is biotinylated from inside. Thus, the internal face of the transporter should be wide enough to allow access to the bulky BM at this position.

We also provide evidence that His110 lies close to the substrate binding/permeation pathway of xCT: 1) BM biotinylation of H110C is blocked by substrates and the nontransportable inhibitor 4-S-CPG (Fig. 5). 2) His110-dependent transport inactivation by MTSES is protected by xCT substrates with an IC50 similar to the Km (Fig. 8). This protection is temperature-independent (Fig. 7), suggesting that no large conformational changes are involved. 3) Replacement of His110 by cysteine (His-H110C) or aspartate (His-H110D) (mimicking LSHATs

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3 C. del Rio, N. Reig, and M. Palacin, unpublished data.
Membrane Topology of xCT

Our results provide experimental support for 12 transmembrane domains for xCT, indicate a re-entrant loop-like structure between transmembrane domains 2 and 3, and identify His410, at the apex facing outside of this re-entrant loop, accessibility to which is restricted by substrates and the inhibitor 4-S-CPG. Functional studies of substituted residues and cysteine accessibility analyses are in progress to gain a more detailed knowledge of the functional key residues and membrane topology of xCT.

Acknowledgment—We thank Robin Rycroft for editorial help.

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Membrane Topology of System Xc⁺ Light Subunit Reveals a Re-entrant Loop with Substrate-restricted Accessibility
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doi: 10.1074/jbc.M402428200 originally published online May 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402428200

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