Two Amino Acid Residues Determine the Low Substrate Affinity of Human Cationic Amino Acid Transporter-2A*

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Mammalian cationic amino acid transporters (CAT) differ in their substrate affinity and sensitivity to trans-stimulation. The apparent $K_m$ values for cationic amino acids and the sensitivity to trans-stimulation of CAT-1, -2B, and -3 are characteristic of system $\gamma^+$. In contrast, CAT-2A exhibits a 10-fold lower substrate affinity and is largely independent of substrate at the trans-side of the membrane. CAT-2A and -2B demonstrate such divergent transport properties, even though their amino acid sequences differ only in a stretch of 42 amino acids. Here, we identify two amino acid residues within this 42-amino acid domain of the human CAT-2A protein that are responsible for the apparent low affinity of both the extracellular and intracellular substrate-binding sites. These residues are located in the fourth intracellular loop, suggesting that they could be part of the translocation pathway. Rather, they may be responsible for the low affinity conformation of the substrate-binding sites. The sensitivity to trans-stimulation is not determined by the same amino acid residues as the substrate affinity and must involve a more complex interaction between individual amino acid residues. In addition to the 42-amino acid domain, the adjacent transmembrane domain X seems to be involved in this function.

The cationic amino acid transporter (CAT) family comprises four members: CAT-1, -2A, -2B, and -3 (for review, see Refs. 1–3). All CAT proteins mediate Na+-independent transport of cationic amino acids. However, they differ in their substrate affinity and sensitivity to trans-stimulation and to pH changes. The transport properties of the mouse and human CAT isoforms (mCAT and hCAT, respectively) have been characterized in greatest detail, e.g. in transport studies in Xenopus laevis oocytes, either using radiolabeled amino acids or by measuring amino acid-induced membrane currents by whole cell voltage clamping. The apparent $K_m$ values for cationic amino acids reported for mouse and human CAT-1, -2B, and -3 (0.1–0.4 mM) are characteristic of system $\gamma^+$ (4–9). In contrast, CAT-2A exhibits a 10-fold lower substrate affinity and also a greater maximal velocity (10, 11). The CAT proteins differ also in their sensitivity to trans-stimulation. The activities of CAT-1, -2B, and -3 are stimulated by physiological concentrations of substrate at the trans-side of the plasma membrane (0.1–1 mM), a characteristic also consistent with system $\gamma^+$. The most pronounced trans-stimulation has been observed for CAT-1. In contrast, transport mediated by CAT-2A is largely independent of the presence of substrate at the trans-side of the membrane. So far, only little information is available about the role of specific CAT protein amino acid residues in the recognition and translocation of cationic amino acids. Glu$^{107}$ has been shown to be essential for the transport activity of mCAT-1 (12). Located in or adjacent to transmembrane domain (TM) III and conserved in all other known CAT isoforms, this Glu residue is likely to be part of the substrate translocation pathway. However, the amino acid residues that determine the particular transport properties of individual CAT isoforms, such as substrate affinity and sensitivity to trans-stimulation, have not been identified. As evidenced from analyses of data bases of both the human and mouse genomes, CAT-2A and -2B are products of the same gene. Two alternative forms of the sixth coding exon give rise to the two splice variants, which differ only in a stretch of 42 amino acids (6, 8, 11). All known CAT proteins exhibit quite similar hydrophobicity plots, suggesting that their structures in the membrane are similar. They are integral membrane proteins with 12–14 putative TMs and intracellular N and C termini. According to the model with 14 TMs, the region divergent between CAT-2A and -2B is located in the fourth intracellular loop and in part of the adjacent TM IX. Interestingly, in this region, the three isoforms exhibiting similar transport properties (CAT-1, -2B, and -3) also show the highest percentage of amino acid sequence identity (3). It is noteworthy that CAT-2A and -2B demonstrate such divergent transport properties, even though their amino acid sequences differ only in 20 residues (within the stretch of 42 amino acids). Replacement of a 27-amino acid fragment containing the corresponding 42-amino acid domain of mCAT-1 with that of mCAT-2A or -2B and vice versa lead to chimERIC PROTEINS WITH TRANSPORT PROPERTIES OF THE DONOR OF THAT DOMAIN (INCLUDING THE APPARENT AFFINITY FOR L-ARGININE AND SENSITIVITY TO TRANS-STIMULATION) (6). In this study, we aimed to identify the amino acid residues in the 42-amino acid domain of hCAT-2A that are responsible for its distinct transport properties.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis and Generation of Chimeric cDNAs—Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, Heidelberg, Germany) and the oligonucleotides listed in Table I. Silent in-frame BamHI and SalI sites were introduced into the coding regions of hCAT-1 and -2A. These sites as well as a Ncol and a KpnI site (both conserved in hCAT-1 and -2A), were used to construct chimeric cDNAs between hCAT-2A and -1. The first letters of the restriction enzymes used are included in the name of each chimera.
e.g. hCAT-2A/1.BK is a chimera that contains the backbone of hCAT-2A and the BamHI/Kpn1 fragment of hCAT-1.

Enhanced Green Fluorescent Protein (EGFP) Fusion Constructs—A construct encoding EGFP fused to the C terminus of hCAT-1 (hCAT-1/EGFP-pSp64T) has been described previously (13). A construct encoding a fusion protein between hCAT-2A and EGFP (hCAT-2A/EGFP-pSp64T) was obtained as described previously for hCAT-2B (13).

Expression of cDNAs in X. laevis Oocytes—All cDNAs were inserted into the BglII site of pSp64T (14). The plasmids were linearized, and cRNA was prepared by in vitro transcription from the SP6 promoter (mMessage mMachine in vitro transcription kit, Ambion, AMS Biotechnology Europe, Cambridgeshire, UK). 30 ng of cRNA (in 36 nl of H2O) were injected into each X. laevis oocyte (Dumont stages V and VI). Oocytes injected with 36 nl of water were used as controls.

Transport Studies in X. laevis Oocytes—l-Arginine uptake was determined 2 days after injection of cRNA as previously described (8). Briefly, oocytes were equilibrated for 2 h at 18 °C in uptake solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 5 mM HEPES, and 5 mM Tris, pH 7.5) containing the indicated concentrations of unlabeled l-amino acids. The oocytes were then transferred to the same solution supplemented with l-[3H]arginine (5–10 μCi/ml; ICN, Eschwege, Germany). After a 15-min incubation (or 1–6 h incubation for steady-state experiments) at 20 °C, the oocytes were washed four times with ice-cold uptake solution and solubilized individually in 2% SDS. The incorporated radioactivity was determined as liquid scintillation count.

Oocytes injected with 36 nl of water were used as controls.

Western Blots—Oocytes were lysed by vortexing in radioimmune precipitation assay buffer (1% deoxycholate, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM MgCl2, 10 mM Tris-HCl, pH 7.2, and 1 mM phenylmethylsulfonyl fluoride) 2 days after injection with wild-type, mutant, or chimeric hCAT-2A cRNA or with water (five oocytes/25 μl of buffer). Lysates were then treated with N-glycosidase F (4 units/25 μl; Roche Applied Science) for 1 h at 37 °C. Radioimmune precipitation assay buffer (75 μl) containing 5 μl of buffer was then added. The samples were spun at 14,000 × g. After determining the protein concentration of the supernatant (using the Bradford reaction, Bio-Rad), an equal volume of sample buffer (125 μl Tris-HCl, pH 6.8, 20% glycerol, 5% SDS, 2% β-mercaptoethanol, 0.001% bromphenol blue, and 1 mM phenylmethylsulfonyl fluoride) was added.

Lysates (20 μg of protein) were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (Protran 88, Schleicher & Schuell, Dassel, Germany). Staining for hCAT-2 proteins was achieved by sequential incubations in Blotto (50 mM Tris-HCl, pH 8, 2 mM CaCl2, 0.1% antifade A (Sigma), 0.05% Tween 20, and 5% nonfat dry milk) containing 10% goat serum for 2 h at room temperature; a 1:100 dilution of anti-hCAT-2 polyclonal antibody in PBS containing 1% bovine serum albumin and 0.1% Tween 20 overnight at 4 °C; three times in Blotto for 15 min at room temperature; a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Calbiochem, Bad Soden, Germany) in Blotto for 1 h at room temperature; three times in 10 mM Tris-HCl, pH 8, 5 mM NaCl, and 0.05% Tween 20; once in 10 mM Tris-HCl, pH 8, and 150 mM NaCl; and finally in chemiluminescence reagent (Renaissance, PerkinElmer Life Sciences, Bad Homburg, Germany) for 1 min. The membranes were then immediately exposed to x-ray films (Agfa, Leverkusen, Germany). For each experiment, two to four different exposure times were used for quantification. Rabbit anti-EGFP peptide polyclonal antibodies (Clontech, Heidelberg, Germany) were used as primary antibodies (1:500) for the detection of EGFP fusion proteins. For standardization, membranes were stripped with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and stained with anti-β-tubulin monoclonal antibody (1:1,000; Sigma) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:3,000; Sigma).

Biotinylation of Cell Surface Proteins—Oocytes were rinsed with ice-cold modified PBS (1.76 mM KH2PO4, 10.1 mM Na2HPO4, and 1.0 mM NaCl), containing 1 mM EGFP peptide polyclonal antibodies (Clontech, Heidelberg, Germany) for 1 min. The membranes were then immediately exposed to x-ray films (Agfa, Leverkusen, Germany). For each experiment, two to four different exposure times were used for quantification. Rabbit anti-EGFP peptide polyclonal antibodies (Clontech, Heidelberg, Germany) were used as primary antibodies (1:500) for the detection of EGFP fusion proteins. For standardization, membranes were stripped with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol for 30 min at 50 °C and stained with anti-β-tubulin monoclonal antibody (1:1,000; Sigma) and peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:3,000; Sigma).

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lengths in hCAT-2A with the corresponding fragments in hCAT-1 (Fig. 1). To this end, we introduced a BamHI and a SalI recognition site in the hCAT-1 and -2A cDNAs without changing the reading frame. We used these sites as well as the conserved NcoI and KpnI sites to exchange fragments of 80 (BamHI/KpnI), 57 (BamHI/SalI), and 46 (NcoI/SalI) amino acids, resulting in the chimeras hCAT-2A/1.BK, hCAT-2A/1.BS, and hCAT-2A/1.NS, respectively. The 80- and 57-amino acid fragments contain the entire region that is divergent between hCAT-2A and -2B, whereas the 46-amino acid fragment lacks the first nine amino acids of the divergent region.

The apparent substrate affinity of the chimeras was determined by expres-

FIG. 1. Models of hCAT-2A and the chimeric and mutant proteins. A, model of hCAT-2A with 14 putative TMs (numbered I–XIV) predicted by the TopPred 2 program (18). The amino acids in the protein region where hCAT-2A differs from hCAT-2B are indicated as closed circles. The positions corresponding to the restriction enzyme recognition sites in the cDNA of hCAT-2A that were used to create chimeric proteins are boxed. These were either already present (NcoI and KpnI sites) or introduced using site-directed mutagenesis (BamHI and SalI sites). The arrows indicate the two point mutations introduced into hCAT-2A. The branched lines indicate putative glycosylation sites. B, sequence comparison of hCAT-1, -2A, -2B, and -3 in the area where hCAT-2A differs from hCAT-2B. Amino acid sequences were aligned using the BLASTp program. The small horizontal brackets mark the positions of restriction enzyme recognition sites in the corresponding cDNAs of hCAT-1 and -2A that were used to create chimeric proteins. Numbers on top refer to the positions of the respective amino acid residues in hCAT-2A. The 42-amino acid domain that differs between hCAT-2A and -2B is marked by vertical brackets. The large horizontal brackets indicate TMs predicted by the TopPred 2 program. The arrows indicate the two most striking differences in the sequence of low affinity hCAT-2A compared with the sequences of high affinity hCAT-1, -2B, and -3. C, schemes of the chimeric and mutant hCAT-2A proteins. The white boxes indicate the hCAT-1 sequence; the gray boxes indicate the sequence common in hCAT-2A and -2B; and the black boxes indicate sequence specific for hCAT-2A.
Substrate Affinity of hCAT-2A

X. laevis oocytes were injected with 36 ng of cRNA (in 36 nl of water) encoding the respective wild-type, chimeric, or mutant hCAT-2A or with 36 nl of water alone. 2 days later, uptake of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 mM L-[3H]arginine was measured over 15 min at 20 °C. The apparent Km values were determined using the Eadie-Hofstee equation (after subtraction of the values obtained with water-injected oocytes). Bars represent means ± S.E. (n = 4–8), with four to six replicates each. Statistical analysis was performed using analysis of variance with Bonferroni’s post-hoc test. ***, **, and *, p < 0.001, 0.01, and 0.05, respectively; ns (not significant), p > 0.05.

In a previous study, we established that, also at the inner side of the membrane, hCAT-2A exhibits a significantly lower apparent substrate affinity than hCAT-1 (8). At saturating extracellular substrate concentrations (Vmax for influx), an apparent steady state is reached when the intracellular substrate-binding sites are also saturated (Vmax for efflux). Cells will therefore accumulate more substrate when expressing a transporter with low substrate affinity at the cytoplasmic side compared with a transporter with high substrate affinity at the

Comparison of the maximal transport activities of hCAT-1/EGFP and hCAT-2A/EGFP. X. laevis oocytes were injected with cRNA encoding hCAT-1 or hCAT-2A with EGFP fused to the C terminus (or with water alone) and analyzed 2 days later. A and B, shown are fluorescent micrographs of cryostat sections (12 μm) from oocytes expressing hCAT-1/EGFP and hCAT-2A/EGFP, respectively. C, the uptake of 10 mM L-[3H]arginine over 15 min at 20 °C was measured, and the values obtained with water-injected oocytes were subtracted. To determine the cell surface expression of the transporters, oocytes were exposed to biotin prior to lysis, and the biotinylated proteins were isolated with streptavidin. The total and cell surface protein expression of each transporter were quantified by Western blotting using a commercial anti-EGFP-antibody. The values for L-arginine transport were then divided by the respective protein values and expressed as the percentage of the values obtained for hCAT-1 (100%). Bars represent means ± S.E. (n = 3–5).

Substrate Affinity of hCAT-2A

Fig. 2. Apparent Km values for L-arginine of wild-type, chimeric, and mutant hCAT-2A proteins in comparison with those of hCAT-1. X. laevis oocytes were injected with 36 ng of cRNA (in 36 nl of water) encoding the respective wild-type, chimeric, or mutant hCAT-2A or with 36 nl of water alone. 2 days later, uptake of 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 mM L-[3H]arginine was measured over 15 min at 20 °C. The apparent Km values were determined using the Eadie-Hofstee equation (after subtraction of the values obtained with water-injected oocytes). Bars represent means ± S.E. (n = 4–8), with four to six replicates each. Statistical analysis was performed using analysis of variance with Bonferroni’s post-hoc test. ***, **, and *, p < 0.001, 0.01, and 0.05, respectively; ns (not significant), p > 0.05.

Comparison of the CAT proteins in the exchanged region revealed two striking differences in the amino acid sequences of hCAT-2A and the high affinity CAT isoforms: an arginine residue (carrying a positive charge) at position 369 of hCAT-2A, where the high affinity isoforms have a negatively charged glutamic acid; and a missing amino acid residue at position 381 of hCAT-2A, where the high affinity isoforms contain an asparagine or histidine residue (Fig. 1B). Therefore, Arg⁹⁶⁹ in hCAT-2A was mutated to Glu, and Asn was inserted in position 381 (hCAT-2A(R369E/N381i)). Transport studies in X. laevis oocytes revealed Km values intermediate between those of hCAT-2A and -1 for the hCAT-2A proteins carrying either single mutation (Fig. 2). The hCAT-2A double mutant exhibited Km values indistinguishable from those of hCAT-1.
cytoplasmic side. The apparent steady-state accumulation under saturating extracellular substrate concentrations thus represents an indirect measurement of the substrate affinity at the intracellular side. To this end, we measured the accumulation of tritiated L-arginine over 6 h in oocytes incubated in isotonic salt solution containing 10 mM L-[3H]arginine (Fig. 3). As observed previously (8), hCAT-2A-expressing oocytes accumulated considerably more L-arginine over the 6-h incubation time than hCAT-1-expressing oocytes (7.2 ± 0.27 versus 1.7 ± 0.13 nmol/oocyte, n = 45). Oocytes expressing the double mutant hCAT-2A(R369E/N381i) accumulated also significantly less L-arginine (3.8 ± 0.23 nmol/oocyte, n = 45) than oocytes expressing hCAT-2A.

Maximal Transport Activity—We routinely observe a higher maximal transport activity in oocytes expressing hCAT-2A compared with those expressing hCAT-1 (5.1 ± 0.88 versus 1.8 ± 0.19 nmol/oocyte/h; p < 0.001). However, it had not been elucidated if this difference between the two proteins is due to differences in the specific activity or rather in the expression level in the plasma membrane. To address this point, we fused EGFP to the C terminus of hCAT-1 and -2A and determined the transport activities and expression levels of the two proteins in *X. laevis* oocytes. Both proteins were predominantly localized to the plasma membrane (Fig. 4, A and B). However, compared with hCAT-1/EGFP, hCAT-2A/EGFP demonstrated a 3-fold higher maximal transport activity (determined at 10 mM L-arginine) in relation to its total and cell surface protein expression (determined in Western blot analyses using anti-GFP antibody) (Fig. 4C). These data indicate that hCAT-2A has indeed a higher maximal transport activity compared with hCAT-1.

We then wondered whether the same protein domain that determines the apparent substrate affinity of hCAT-2A also determines its maximal transport activity. To quantify the protein expression levels of wild-type and mutant hCAT-2A proteins, we generated immune plasma against the C terminus protein expression levels of wild-type and mutant hCAT-2A proteins. We then wondered whether the same protein domain that determines its maximal transport activity. To quantify the protein expression levels of wild-type and mutant hCAT-2A proteins, we generated immune plasma against the C terminus of hCAT-1 and -2A and determined the level in the plasma membrane. To address this point, we fused EGFP to the C terminus of hCAT-1 and -2A and determined the

**Fig. 5.** Protein expression of wild-type, chimeric, and mutant hCAT-2A proteins. Western blotting was performed with lysates from oocytes prepared 2 days after injection of cRNA from one of the indicated transporters or of water alone. Protein (20 μg/lane) was separated by 10% SDS-PAGE and blotted on to a membrane, and the membrane was incubated with affinity-purified anti-hCAT-1 antibody (A and B, upper panels). Lysates in A were treated with N-glycosidase F. Black and white arrows indicate the glycosylated and deglycosylated hCAT-2A proteins, respectively. The blots were then stripped and incubated with anti-β-tubulin monoclonal antibody for standardization (A and B, lower panels).
Substrate Affinity of hCAT-2A

Fig. 6. Assessment of the specific transport activities of wild-type, chimeric, and mutant hCAT-2A proteins. A, the maximal transport activity of each transporter was calculated from the experiments described in the legend to Fig. 2. Bars represent means ± S.E. (n = 4–8), with four to six replicates each. B, protein expression was quantified by densitometry of several Western blots. For standardization, values obtained for each transporter were divided by the respective protein values in hCAT-2A and expressed as the percentage of the values obtained for hCAT-2A (100%).

Our data demonstrate that a fragment of 80 amino acids extending from the end of TM VIII to the end of TM X (Fig. 1A) can confer the transport properties of hCAT-1 to hCAT-2A. This fragment contains the protein domain of 42 amino acids corresponding to the domain divergent between hCAT-2A and -2B (comprising the fourth intracellular loop and part of TM IX) (Fig. 1, A and B). The reciprocal chimera (hCAT-1/2A.BK) carrying the 80 amino acids from hCAT-2A in the hCAT-1 backbone had the transport properties of hCAT-2A (data not shown). This is in agreement with previous results from mCAT proteins (6). Also smaller fragments containing only 57 or 46 amino acids of hCAT-1 could transfer the high substrate affinity and sensitivity to trans-stimulation of hCAT-1 to hCAT-2A. Interestingly, the reciprocal chimeras had very low transport activities (data not shown), suggesting important intramolecular interactions within the 80-amino acid fragment. The two hCAT-1 chimeras carrying 57 and 80 amino acids of hCAT-2A, respectively, differ only in three amino acid residues located in TM X (Fig. 1A). These sequence changes in TM X of hCAT-2A or -2B versus hCAT-1 are conservative (Fig. 1B). However, the particular residues are conserved in the respective orthologs of different mammalian species, suggesting that they might be crucial for the transport function. This is also demonstrated by the reduced efflux activity of the hCAT-2A/1.SK chimera, which differs from hCAT-2A only in these three residues (Table II).

Mutation of two amino acid residues (Arg<sup>369</sup> to Glu and insertion of Asn<sup>381</sup>) was sufficient to decrease the apparent <i>K<sub>m</sub></i> of hCAT-2A at the extracellular face to values similar to those of hCAT-1. The localization of the two residues in an intracellular loop (as opposed to a TM) suggests that they are not part of the substrate-binding site, but influence substrate binding indirectly. The reciprocal mutant of hCAT-1 (hCAT-1(E367R/N381i)) had no transport activity (data not shown), demonstrating that the two amino acid residues (Glu<sup>369</sup> and Asn<sup>379</sup>) are essential for transport function in the hCAT-1 backbone. It seems that CAT-2 is more flexible in accepting changes in the amino acid sequence, probably because it can naturally accommodate both a low and a high affinity domain. Our steady-state experiments revealed that the two mutations in hCAT-2A also increased the apparent affinity of the intracellular substrate-binding site. Using high pressure liquid chromatographic analyses, we have previously established that hCAT-2A-expressing oocytes exhibit higher l-arginine levels than hCAT-1-expressing oocytes when incubated in high extracellular l-arginine concentrations (8). Therefore, the larger accumulation of tritiated l-arginine in hCAT-2A-expressing oocytes versus hCAT-1-expressing and hCAT-2A(R369E/N381i)-expressing oocytes observed here indeed reflects higher intracellular l-arginine levels that must be reached before efflux can occur at a maximal rate. The simultaneous change in the affinity of the intracellular and extracellular substrate-binding sites further supports the concept of an indirect influence of the two residues on substrate binding.

Using fusion proteins between hCAT-1 or hCAT-2A and EGFP, we demonstrated that the two CAT isoforms differ indeed in their maximal transport activities. The ratio of transport activity to protein expression stayed about the same irrespective of whether the total protein expressed or only the protein expressed at the cell surface was taken into consideration. The EGFP fusion partner did not seem to influence the transport properties of the hCAT proteins, as the <i>K<sub>m</sub></i> and <i>V<sub>max</sub></i> values obtained for the hCAT fusion proteins were not different from those determined for the native hCAT isoforms (Ref. 13 and data not shown). In influx and efflux experiments, all hCAT-2A/1 chimeras had maximal activities similar to those of hCAT-2A. Therefore, the specific transport activities of the hCAT proteins do not seem to be controlled by the same protein domain that determines the apparent substrate affinity and the sensitivity to trans-stimulation. Our results with the hCAT chimeras are in contrast to our earlier study carried out with chimeras of mCAT-1, -2A, and -2B, where the <i>V<sub>max</sub></i> values obtained for each chimera were very similar to the <i>V<sub>max</sub></i> values obtained for the respective donor of the 80-amino acid fragment.
pressed in section was examined in efflux experiments. Each transporter was examined in efflux experiments. Each transporter was examined in efflux experiments. The hCAT-2A backbone has been truncated with water-injected oocytes (766 ± 91 dpm). Shown are the means ± S.E. (n) obtained in the efflux experiments described in the legend to Fig. 6 after subtracting the values obtained with water-injected oocytes (766 ± 91 dpm).

| L-[3H]Arginine efflux at 1 mM extracellular L-arginine | Efflux |
|------------------------------------------------------|--------|
| hCAT-2A                                              | 11,369 ± 5410 (6) |
| hCAT-2A/1BK                                          | 11,291 ± 643 (4)  |
| hCAT-2A/1BS                                          | 6934 ± 2575 (3)  |
| hCAT-2A/1INS                                         | 8144 ± 1933 (3)  |
| hCAT-2A/R369E                                        | 1651 ± 113 (5)   |
| hCAT-2A/(R369E)                                      | 3124 ± 660 (6)   |
| hCAT-2A/(R369E/N381i)                                | 3051 ± 552 (5)   |
| hCAT-2A/1.NK                                         | 6336 ± 746 (4)   |
| hCAT-2A/1.SK                                         | 1900 ± 386 (8)   |
| hCAT-2A/1.SK(R369E)                                  | 1844 ± 401 (8)   |
| hCAT-1                                               | 3101 ± 648 (5)   |

(6). However, in these studies, we did not control for protein expression and therefore could not determine the specific activity of each transporter. The reduced transport activity of the double mutant hCAT-2A(R369E/N381i) in the influx experiments likely resulted from an interference with the overall protein structure, e.g. the reciprocal mutant had no activity. Interestingly, in efflux experiments, the activity of the double mutant was higher than that of hCAT-2A/R369E, whereas the opposite was observed in influx experiments. This demonstrates that the two transport pathways are not necessarily coupled.

The lack of trans-stimulation indicates that a transporter can move between the outward and inward facing conformation without substrate, therefore mediating net transport. In contrast, a strongly trans-stimulated transporter works only in the exchange mode. In our previous work, we extensively characterized the trans-stimulation of CAT-1 and -2A, demonstrating that both transporters work symmetrically, e.g. hCAT-1-mediated influx and efflux are markedly reduced at low concentrations of trans-substrate, whereas influx and efflux mediated by hCAT-2A are unchanged (10). In the present study, we performed efflux experiments to determine the sensitivity of the chimeric proteins to trans-stimulation because the concentration of cationic amino acids can be defined more precisely in the extracellular buffer compared with the cytosol. The concentration of 1 mM trans-L-arginine was chosen for these experiments because our previous results demonstrated that trans-stimulation of hCAT-1 is saturated at 10 μM L-arginine and that hCAT-2A is largely independent of extracellular L-arginine up to 10 mM (6, 8). Our results with the hCAT-2A/1.BK and hCAT-2A/1.NK chimeras demonstrate that the 69-amino acid segment encoded by the NcoI fragment does not seem to contribute. However, we were not able to pinpoint the amino acid residues within the 69-amino acid fragment that are responsible for the sensitivity to trans-stimulation. The results with the hCAT-2A/1.SK chimera (carrying TM X of hCAT-1 in the hCAT-2A backbone) suggest that TM X has an influence on the sensitivity of hCAT-2A to trans-stimulation. However, hCAT-2A/1.SK and hCAT-2A/(R369E) both exhibited an increased sensitivity to trans-stimulation compared with hCAT-2A, but the sensitivity of hCAT-2A/1.SK(R369E) was not further increased. Also, the sensitivity of the double mutant hCAT-2A/(R369E/N381i) to trans-stimulation indicates that a transporter can move between the outward and inward facing conformation without substrate, therefore mediating net transport. In contrast, a strongly trans-stimulated transporter works only in the exchange mode. In our previous work, we extensively characterized the trans-stimulation of CAT-1 and -2A, demonstrating that both transporters work symmetrically, e.g. hCAT-1-mediated influx and efflux are markedly reduced at low concentrations of trans-substrate, whereas influx and efflux mediated by hCAT-2A are unchanged (10). In the present study, we performed efflux experiments to determine the sensitivity of the chimeric proteins to trans-stimulation because the concentration of cationic amino acids can be defined more precisely in the extracellular buffer compared with the cytosol. 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Also, the sensitivity of the double mutant hCAT-2A/(R369E/N381i) to trans-stimulation is significantly lower than that of hCAT-2A. The lack of trans-stimulation indicates that a transporter can move between the outward and inward facing conformation without substrate, therefore mediating net transport. In contrast, a strongly trans-stimulated transporter works only in the exchange mode. In our previous work, we extensively characterized the trans-stimulation of CAT-1 and -2A, demonstrating that both transporters work symmetrically, e.g. hCAT-1-mediated influx and efflux are markedly reduced at low concentrations of trans-substrate, whereas influx and efflux mediated by hCAT-2A are unchanged (10). In the present study, we performed efflux experiments to determine the sensitivity of the chimeric proteins to trans-stimulation because the concentration of cationic amino acids can be defined more precisely in the extracellular buffer compared with the cytosol. 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Also, the sensitivity of the double mutant hCAT-2A/(R369E/N381i) to

significant, p < 0.05. B, shown are schemes of the additional chimeras formed in efflux experiments. The hCAT-2A backbone has been truncated as indicated by the slanted lines. C, trans-stimulation experiments with the new chimeras were performed as described for A.
trans-stimulation was not different from that of hCAT-2A, suggesting that the insertion of the asparagine residue desensitizes the protein to trans-stimulation. A shortcoming of these experiments lies in the low efflux activities of hCAT-2A/1.SK and all hCAT-2A mutants, making the interpretation of these results difficult, as a small reduction in the absolute efflux rate at no trans-substrate is sufficient to suggest an increased sensitivity to trans-stimulation. The hCAT-2A(R369E/N381i) mutant, which exhibited the same substrate affinity as hCAT-1, but no sensitivity to trans-stimulation, demonstrates that substrate affinity and sensitivity to trans-stimulation can be separated.

In summary, our study provides important insights into the structure/function relation of the hCAT proteins. We have identified two amino acid residues that determine the apparent substrate affinity of CAT-2A on both sides of the membrane. These residues are not localized within a transmembrane domain and therefore are not likely to be part of the substrate-binding site and translocation pathway. Their localization in the fourth intracellular loop suggests that this protein domain might control the affinity of the substrate-binding sites indirectly, e.g., by influencing their conformation. The two amino acid residues are within the 42-amino acid fragment where the sequence of hCAT-2A diverges from the sequence of hCAT-2B and that extends into TM IX. This fragment also influences the sensitivity of hCAT-2A to trans-stimulation. However, other amino acid residues and a more complex interaction between different amino acid residues within this area must be involved. In addition, the adjacent TM X seems to also play a role in determining the sensitivity to trans-stimulation. These findings, together with the observation that subtle changes in the sequence of TM X lead to loss of function in hCAT-1, led us to assume that TMs IX and X might well be part of the translocation pathway.

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