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Structure and Function of Cationic Amino Acid Transporters (CATs)

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Abstract. The CAT proteins (CAT for cationic amino acid transporter) are amongst the first mammalian amino acid transporters identified on the molecular level and seem to be the major entry path for cationic amino acids in most cells. However, CAT proteins mediate also efflux of their substrates and thus may also deplete cells from cationic amino acids under certain circumstances. The CAT proteins form a subfamily of the solute carrier family 7 (SLC7) that consists of four confirmed transport proteins for cationic amino acids: CAT-1 (SLC7A1), CAT-2A (SLC7A2A), CAT-2B (SLC7A2B), and CAT-3 (SLC7A3). SLC7A4 and SLC7A14 are two related proteins with yet unknown function. One focus of this review lies on structural and functional differences between the different CAT isoforms. The expression of the CAT proteins is highly regulated on the level of transcription, mRNA stability, translation and subcellular localization. Recent advances toward a better understanding of these mechanisms provide a second focus of this review.

Key words: Amino acid starvation — Amino acid responsive element — Cationic amino acid transporter — IRES — micro RNA — Nitric oxide — Paraspeckles — SLC7 — System y^+

Abbreviations: CAT, cationic amino acid transporter, prefix h, r, m: human, rat and mouse, respectively; HAT, heteromeric amino acid transporter; DC, dendritic cell; HEK, human embryonic kidney cells; IRES, internal ribosomal entry sequence; NOS, nitric oxide synthase, prefix e, and i: endothelial and inducible isoform, respectively; PAEC, porcine aortic endothelial cells; PKC, protein kinase C; SLC, solute carrier family; TM, transmembrane domain.

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Cationic Amino Acid Transporters (CATs) — System y^+ and More

The family of cationic amino acid transporters (CAT) comprises four members, CAT-1, -2A, -2B and -3, which all exhibit a nearly identical substrate pattern for cationic L-amino acids (Closs, 2002; Deves & Boyd, 1998). It constitutes a subfamily of the solute carrier family 7 (SLC7). CAT-2A and -2B are splice variants that differ only in a stretch of 42 amino acids (Closs et al., 1993a; Closs et al., 1997; Closs et al., 1993c; Kakuda et al., 1993; Kavanaugh et al., 1994). In contrast, CAT-1 and CAT-3 are encoded for by separate genes (Albritton et al., 1993a; Hosokawa et al., 1997; Ito & Groudine, 1997; Kim et al., 1991; Wang et al., 1991; Yoshimoto, Yoshimoto & Meruelo, 1991). The human genome organization (HUGO) has assigned the gene names SLC7A1, A2 and A3 to human CAT-1, CAT-2, and CAT-3, respectively (Verrey et al., 2004). An additional protein, designated SLC7A4 or CAT-4, has also been allocated to the CAT branch of the SLC7 family. However, in spite of its localization in the plasma membrane, SLC7A4 does not exhibit any transport activity for cationic, neutral or acidic amino acids when over-expressed in various human cells or in oocytes from *Xenopus laevis* (Wolf et al., 2002). Its physiological function remains thus elusive. The second branch of the SLC7 family comprises the so-called light chains of heteromeric amino acid transporters (HATs, SLC7A5-11). In contrast to the CAT proteins, these transporters need to associate with a glycoprotein (SLC3A1 or A2) to be targeted to the plasma membrane (Verrey et al., 2004). They are therefore also referred to as glycoprotein-associated transporters. SLC7A12 and 13 are assigned to the HAT light chain subfamily, although so far glycoproteins that may target them to the plasma membrane have not been identified. The two SLC7 subfamilies differ also in their predicted membrane topology, with the CAT and the glycoprotein-associated transporters exhibiting 14 and 12 putative transmembrane domains

(TMs), respectively. SLC7A14 is the youngest family member with yet unknown function. Although it has 14 predicted TMs and a closer sequence identity to the CAT than to the HAT light chain subfamily, it exhibits an intracellular localization when expressed in a variety of human cell lines (JP Boissel, unpublished observation).

All known CAT proteins transport cationic L-amino acids in a Na^+-independent way. The transport properties and expression pattern of CAT-1 resemble strongly that of system y^+, described as a widely expressed, Na^+-and pH-independent, cationic amino acid-preferring transport activity (White & Christensen, 1982; White, Gazzola & Christensen, 1982; Deves & Boyd, 1998). Like system y^+, CAT-1 exhibits K_M values for L-arginine, L-lysine and L-ornithine of 100–150 μM, and is strongly stimulated by substrate at the *trans*-side of the membrane (Closs et al., 1997; Kim et al., 1991). It thus works preferably as an exchanger. Also like system y^+, membrane hyperpolarization activates CAT-1–mediated substrate influx (Bussolati et al., 1989; Kavanaugh, 1993). CAT-2B and CAT-3 show a somewhat lower apparent substrate affinity and are less dependent on *trans*-stimulation (Closs et al., 1997; Vekony et al., 2001). In addition, transport through CAT-2B decreases at pH levels lower than 7.5, while transport through CAT-1 and CAT-3 stays largely the same between pH 5.5 and 8.5 (Closs et al., 1997; Vekony et al., 2001). Although CAT-1 seems to conform best to system y^+, CAT-2B and CAT-3 are also considered as system y^+-like transporters. The differences between these CAT isoforms can probably only be reliably detected when each transporter is expressed individually in the same expression system and at a comparable level. In contrast, the activity of CAT-2A can clearly be distinguished from the system y^+ transporters. Even though it differs only in a stretch of 42 amino acids from CAT-2B, CAT-2A exhibits an about tenfold lower substrate affinity and is relatively insensitive to *trans*-stimulation (Closs et al., 1993a; Closs et al., 1997; Kavanaugh et al., 1994). Using radiolabeled amino acids in flux studies with *Xenopus laevis* oocytes, we did not observe any transport activity of the CAT proteins for neutral or anionic amino acids (Closs et al., 1993a; Kim et al., 1991; Vekony et al., 2001). L-Histidine is a substrate for hCAT-1 at low pH, when it is predominantly protonated (Kim et al., 1991). This is consistent with earlier observations on Ehrlich ascites tumor cells, showing Na^+-independent inhibition of histidine transport by cationic amino acids to increase with decreasing pH (Im & Christensen, 1976). In contrast, hCAT-3 does not recognize the protonated histidine as a substrate (Vekony et al., 2001). This is the only difference in substrate recognition between the CAT isoforms we could identify so far. However, by means of either electrophysiological or competition studies, others found an interaction of neutral and even anionic amino acids with the CAT proteins (Wang et al., 1991; Hosokawa et al., 1997; Ito & Groudine, 1997). As reported for system y^+, CAT proteins seem to have a higher affinity for cationic amino acids with a long carbon backbone: homoarginine > arginine, lysine > ornithine > 2,4–diamino-*n*-butyric acid (Deves & Boyd, 1998; Ito & Groudine, 1997; Kakuda et al., 1993; Kavanaugh et al., 1994; Vekony et al., 2001; White & Christensen, 1982; White et al., 1982).

The CAT proteins are not the only transporters that mediate Na^+-independent cationic amino acid transport. The HATs 4F2hc/y^+LAT1 and 4F2hc/y^+LAT2 (SLC3A2/SLC7A7 and SLC7A6) and rBAT/$b^{0,+}$AT (SLC3A1/SLC7A9) accept cationic as well as neutral amino acids. They mediate system y^+L and $b^{0,+}$ transport activity, respectively. Transport of neutral amino acids by the former is Na^+-dependent, by the latter, Na^+-independent (Van Winkle, Campione & Gorman, 1988; Deves, Chavez & Boyd, 1992; Torrents et al., 1998; Borsani et al., 1999; Broer et al., 2000; Feliubadalo et al., 1999, Pfeiffer et al., 1999a, 1999b;). Endogenous CAT-mediated transport of cationic amino acids can thus be distinguished from transport through other systems as the component resistant to inhibition by large neutral amino acids (such as leucine) in the presence of Na^+. In addition, system y^+ (CAT), but not y^+L (4F2hc/y^+LAT), is sensitive to inhibition by N-ethylmaleimide (NEM). The two transport systems, which are co-expressed in many cell types, can thus be distinguished by their differential sensitivity to this sulfhydryl reagent (Deves, Angelo & Chavez, 1993). So far it has not been determined if NEM inhibits all CAT isoforms and the cysteine residues in the CAT proteins sensitive to NEM have not yet been identified.

Structure/Function Analysis of the CAT Proteins

The CAT proteins are predicted to have 14 TMs and intracellular N-and C-termini (Fig. 1*A*) (Albritton et al., 1989). Although alternative models with 12 or 13 TMs have been suggested, several experimental data support the 14-TM model. The third extracellular loop of mouse CAT-1 serves as a binding site for ecotropic murine leukemia viruses and thus has been confirmed to be positioned at the outside (Albritton et al., 1993b). In addition, two asparagine residues (223 and 229) in that loop have been shown to be glycosylated (Kim & Cunningham, 1993). In contrast, Asn^{373} in a putative glycosylation site located intra- and extracellularly according to the 14-TM and 12-TM model, respectively, is not glycosylated and thus most likely intracellular. Similarly, a consensus site for N-glycosylation at the C-terminus of human CAT-2A is not glycosylated, indicating that the

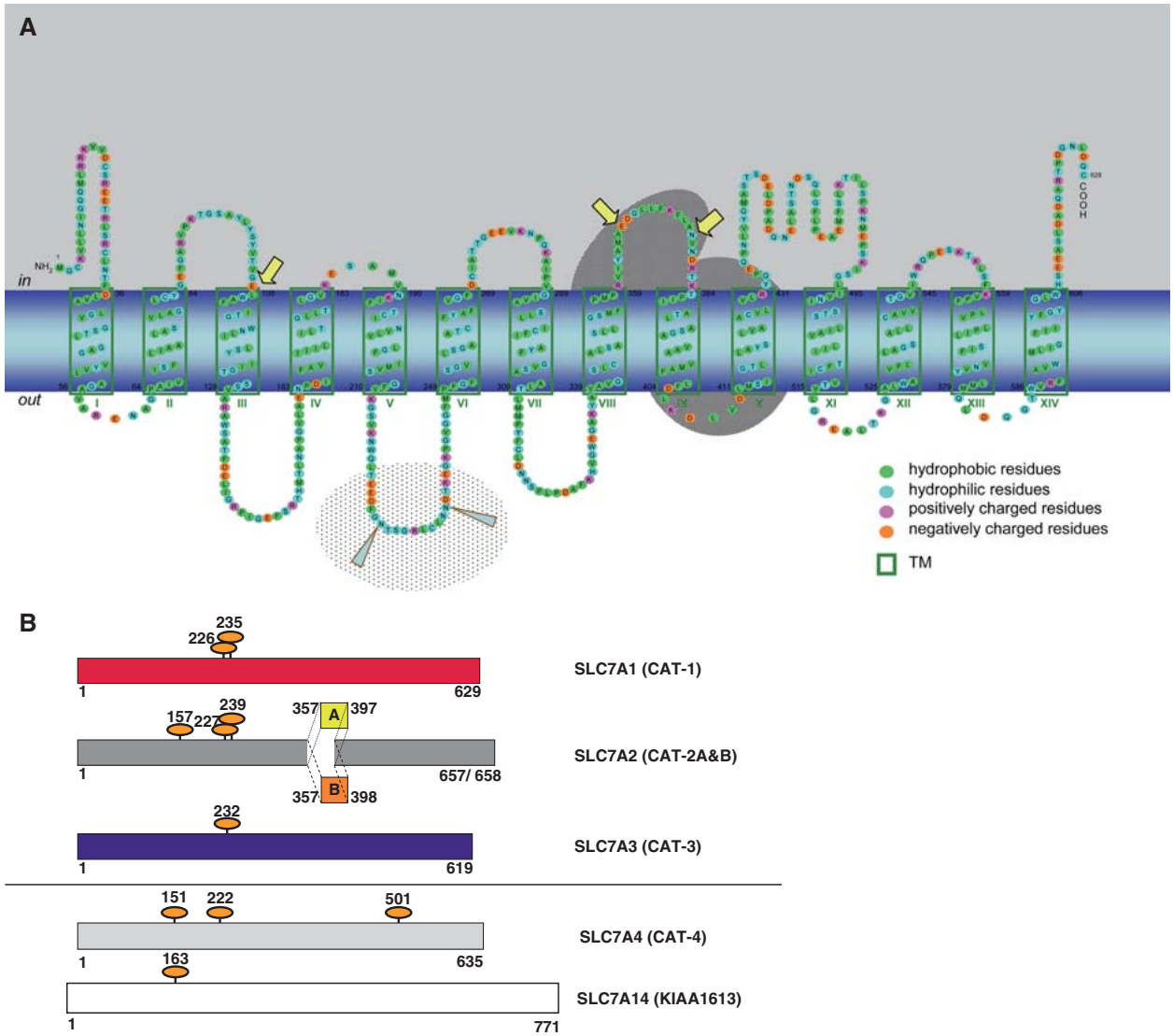

Fig. 1. SLC7 family members with 14 putative transmembrane domains (TM). (*A*) Snake model of hCAT-1: the yellow arrows point to residues identified to be important for transport function. The white wedges indicate the glycosylation sites. The protein region exchanged between CAT-1 and CAT-2A that confers the transport properties of the respective donor of that region is marked with gray ovals. *See* text for more detail. (*B*) Schemes of all the SLC7 family members with putative 14 TMs. The orange ovals indicate putative glycosylation sites. So far, transport function has only been shown for CAT-1, −2A, −2B, and −3.

C-terminus is also intracellular (P. Gräf, 1998, doctoral thesis, Johannes Gutenberg University, Mainz, Germany). Immunostaining of unpermeabilized cells with antibodies directed against the third and fourth extracellular loop of mCAT-1 have also confirmed the extracellular position of the respective protein region (Woodard et al., 1994).

Mutation analysis has shown that glycosylation of the third extracellular loop has no influence on the transport properties of mCAT-1 (Kim & Cunningham, 1993; Wang et al., 1996). This region of the CAT-1 protein exhibits great variability between different species and even amongst individual mouse strains (where CAT-1 serves as a virus receptor), indicating a co-evolution of virus and host proteins (Wang et al., 1996). In spite of this variability, N-glycosylation in this loop of CAT-1 is well conserved. With the exception of SLC7A14, all other 14-TM isoforms of the SLC7 family contain either one (hCAT-3 and the orphan SLC7A4) or two (hCAT-2A and B) N-linked glycosylation sites in this loop (Fig. 1*B*). Additional glycosylation sites are predicted in the second extracellular loop of hCAT-2A and B, SLC7A4 and SLC7A14 and in the 6th loop of SLC7A4, but these sites have not been confirmed experimentally.

Amino acid residues essential for transport function of the CAT proteins have been identified through mutation analysis. The conservative mutation of Glu^{107} to Asp in mCAT-1 leads to loss of

transport activity, while cell membrane targeting and function as a virus receptor are preserved (Wang, Kavanaugh & Kabat, 1994). This suggests that Glu^{107} located at the intracellular face of the third TM of mCAT-1 may be part of the substrate transition pore. This residue is conserved in all 14-TM SLC7 members as well as in homologues from yeast, but not in the HAT light chains. The divergent transport properties of CAT-2A and -2B imply that the stretch of 42 amino acids where the two carriers differ from each other is a crucial determinant of the transport properties. The three isoforms exhibiting similar transport properties (CAT-1, -2B and -3) also show the highest percentage of amino acid sequence identity in that region. In fact, replacement of a protein fragment containing the corresponding region of mCAT-1 by that of mCAT−2A or −2B (and vice versa) leads to chimeric proteins with apparent substrate affinities and sensitivities to trans-stimulation of the respective donor of that region (Closs et al., 1993c). According to the 14-TM model, this region comprises the fourth intracellular loop and part of the adjacent TMs. Replacement of two amino acid residues within intracellular loop 4 of human CAT-2A by the corresponding residues of hCAT-1 confers apparent high substrate affinity to hCAT-2A, both at the outward- and inward-facing substrate binding site (Habermeier et al., 2003). Interestingly, the reciprocal hCAT-1 mutant is not functional, indicating important interactions of these residues with the transporter backbone that were interrupted by the mutation.

The 14-TM SLC7 proteins exhibit only a few polymorphisms, suggesting that the proper function of these proteins does not tolerate many alterations in the protein sequence. The NCBI (National Center for Biotechnology Information) data base lists only silent single nucleotide polymorphisms (SNP) for the coding region of human CAT-1 and no SNPs for SL7A4 (Table 1). Variations in 1, 2 and 3 amino acid residues are known for SLC7A14, hCAT-3 and hCAT-2A and B, respectively.

Table 1. Number of single nucleotide polymorphism (SNP) in the coding region of the human CAT isoforms reported by the NCBI data base: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=snp

hCAT isoform	Number of cSNPs	Number of altered amino acid residues (position-alternative residues)
hCAT-1	4	0
hCAT-2A&B	3	3 (20V/M; 37M/L; 547L/Q)
hCAT-3	3	2 (191K/R; 508L/V)
SLC7A4	0	0
SLC7A14	4	1 (330G/R)

Physiological Function of the CAT Proteins

CAT-, that is, system y^+-mediated transport, seems to be the major entry pathway for cationic amino acids in non-epithelial cells. In contrast, the apically localized systems $b^{0,+}$, and $B^{0,+}$ (a Na^+-dependent transporter for cationic and neutral amino acids) act in concert with the basolateral system y^+L to mediate the vectorial transport of cationic amino acids through epithelial cells that is necessary for efficient absorption/reabsorption (Bauch et al., 2003). System y^+L transporters are also expressed in many non-epithelial cells where they also function most likely as an export rather then an import pathway for cationic amino acids under physiological conditions.

Cationic amino acid supplied through the CAT proteins feeds into protein synthesis and other enzymatic reactions dependent on these amino acids, including the synthesis of nitric oxide (NO), urea, creatine and agmatine from arginine or the synthesis of polyamines, proline and glutamine from ornithine. There is increasing evidence that CAT-mediated transport can be an important determinant of these processes (Mann, Yudilevich & Sobrevia, 2003; Closs et al., 2004; San Martin & Sobrevia, 2006).

The generation of mice with deletions of individual CAT isoforms is a crucial approach for a better understanding of the roles the respective isoforms play in cationic amino acid supply in different cell types. Homozygous deletion of the CAT-1 gene in mice is lethal (Perkins et al., 1997). The knockout mice exhibit a 25% reduction in size compared to their wild-type littermates, suffer from severe anemia and die on day one after birth. In contrast, heterozygous CAT-1 knockout mice exhibit no conspicuous phenotypical abnormalities. The relatively normal development of the homozygous CAT-1 knockout mice before birth is probably due to the expression of CAT-3 during embryogenesis and fetal development (Ito & Groudine, 1997; Nicholson et al., 1998).

Genetic ablation of CAT-2, which affects both splice variants, leads to a decrease in sustained NO production by the inducible nitric oxide synthase in peritoneal macrophages and astrocytes, but not in fibroblasts (Nicholson et al., 2001; Manner, Nicholson & MacLeod, 2003; Nicholson, Manner & MacLeod, 2002). A recent report found that these mice exhibit baseline inflammation and an increase in dendritic cell (DC) activation in their lungs in the absence of identifiable pathogenic stimuli (Rothenberg et al., 2006). In addition, NO production in alveolar macrophages of these mice was reduced. This suggests that in the lung, CAT-2(B) may indirectly be involved in the suppression of inflammation by ensuring arginine supply to inducible nitric oxide synthase (iNOS) in macrophages for the synthesis of NO known to suppress DC activation. In the CAT-2-deficient mice reduced NO production may thus lead to an unopposed DC activation. Surprisingly, the

lack of CAT-2A, which is highly expressed in the liver, seems to be coped with quite well by these mice. We have proposed that the low-affinity, high-capacity CAT-2A serves to remove surplus cationic amino acids from the portal circulation (Closs et al., 1993a). It would thus be interesting to see if the knockout mice tolerate a diet with high cationic amino acid content. Also, the physiological significance of the large increase in CAT-2A expression observed in skeletal muscle after surgical trauma (Kakuda et al., 1998) could be studied in the knock-out animals.

For CAT-3, SLC7A4, and SLC7A14, knock-out animals have not been established so far. Given the wide expression of CAT-3 in embryonic tissues, total ablation of this gene would most likely be fatal at an early stage due to interference with normal development. Conditional and tissue-specific deletion of the individual CAT isoforms is needed to elucidate their specific function in cellular processes dependent on cationic amino acids, such as NO and polyamine synthesis. Alternatively antisense or small interfering RNAs can be used to down-regulate individual CAT isoforms. Such an approach has been used to demonstrate the importance of CAT-1 in NO synthesis in the renal medulla (Kakoki, Wang & Mattson, 2002).

Complex Regulation of CAT Expression

With the exception of the liver, CAT-1 is almost ubiquitously expressed, although in varying abundance. In contrast, the two CAT-2 splice variants exhibit a much more restricted expression pattern. CAT-2A is strongly expressed in the liver and rather weakly in skeletal muscle and pancreas. CAT-2B can be induced by pro-inflammatory cytokines and bacterial polysaccharide in a variety of cell lines, often together with arginine-consuming enzymes such as arginase and NOS. CAT-3 is widely expressed in the developing mouse embryo, but seems to be confined to the brain in adult animals. In humans, CAT-3 expression has also been found in peripheral tissues such as thymus and mammary gland. Extensive studies on the expression and regulation of CAT mRNAs and arginine transport in various cell types have been published and reviewed recently (MacLeod, 1996; Closs, 2002; Closs & Rotmann, 2005). We focus here on recent reports demonstrating an extensive regulation of CAT-1 and CAT-2 expression by different kinds of stress, e.g., nutrient deprivation (Fig. 2). Both genes produce very large transcripts of about 4.5 and 8 kb (with the coding region being less than 2 kb) suggestive of posttranscriptional regulation. Hatzoglou's group observed that amino acid starvation increases not only transcription, but also mRNA stability and translation of CAT-1 mRNA in rat C6 glioma cells (Hatzoglou et al., 2004). Transcription of CAT-1 mRNA occurs from a TATA-less promoter that extends into the first exon of the rCAT-1 mRNA. An amino acid-responsive element located in the first exon is required for the stimulation of transcription (Fernandez et al., 2003). In addition, activation of GCN2 kinase (which phosphorylates the eukaryotic initiation factor 2α, eIF2α) is necessary for transcriptional activation, suggesting the involvement of newly translated transcription factors in the induction of rCAT-1 transcription. The starvation-induced increase in the stability of the rCAT-1 mRNA is dependent on the presence of an AU-rich element (ARE) within a 217 bp fragment in the distal part of the rCAT-1 3′UTR that binds to the ELAV (embryonic lethal abnormal vision) protein HuR (Yaman et al., 2002).

Translation of rCAT-1 mRNA under amino acid starvation is initiated from an internal ribosomal entry sequence (IRES) within the 5′UTR of the CAT-1 mRNA (Fernandez et al., 2001). Efficient translation through the IRES also requires the phosphorylation of eIF2α by either GCN2, PKR-like ER kinase (PERK) or double-stranded RNA-dependent protein kinase (PKR) (Fernandez et al., 2002a, 2002c). The regulated activity of the IRES depends on the translation of an upstream open reading frame (μORF of 48 amino acids) contained in the 5′UTR of the rCAT-1 mRNA that overlaps with the IRES (Fernandez et al., 2002b; Yaman et al., 2003). Ribosome-stalling in the upstream ORF induced either by cycloheximide or by the introduction of rare codons stimulates translation of the downstream CAT-1 ORF (Fernandez et al., 2005).

A completely different mechanism of translational control has recently been discovered for human CAT-1 in Huh7 hepatoma cells expressing the liver-specific microRNA miR-122 (Bhattacharyya et al., 2006). MicroRNAs are known to imperfectly base-pair with the 3′UTR of target mRNAs and to prevent protein accumulation by either repressing translation or inducing mRNA degradation. The human CAT-1 3′UTR contains several potential target sites for miR-122. Insertion of these sites into the 3′UTR of a chimeric mRNA has detrimental effect on its translation and stability (Chang et al., 2004). However, the endogenous hCAT-1 mRNA and reporters bearing its 3′UTR are relieved from the miR-122-induced inhibition by different stress conditions, including amino acid deprivation (Bhattacharyya et al., 2006). De-repression of the CAT-1 mRNA is accompanied by its release from cytoplasmic processing bodies (P-bodies). Its recruitment to polysomes requires binding of HuR to the 3′UTR at sites that do not correspond to the ARE responsible for stabilizing rCAT-1 (*see* above and Fig. 2). Reporter assays demonstrate that miR-122-induced repression and stress-induced de-repression are both mediated by the 3′UTR and hence do not involve an IRES. Also, starvation-induced hCAT-1 translation in Huh7 cells

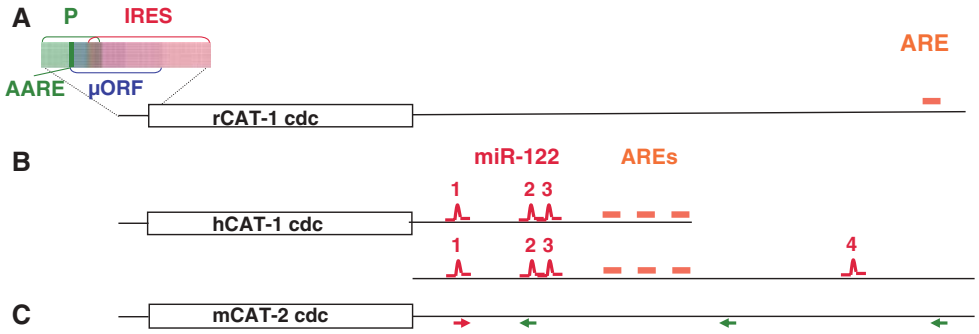

Fig. 2. Translational regulation of CAT expression**.** The scheme depicts different mechanisms involved in the regulation of the expression of rat CAT-1 (*A*), human CAT-1 (*B*) and mouse CAT-2 (*C*). Both, CAT-1 and CAT-2 have large (about 8 kb) and smaller (about 4.5 kb) messages and alternative 5′UTRs (*not shown*). (*A*) The rCAT-1 mRNA contains overlapping elements in its 5′UTR: a promoter element (*P*) including an amino acid-responsive element (AARE), an upstream open reading frame (μORF) and an internal ribosomal entry side (IRES). An AU-rich element (ARE) that binds the ELAV protein HuR is located in the distal part of the 3′UTR. Activation of rCAT-1 expression by amino acid deprivation involves increased transcription, mRNA stability and translation. The latter is brought about by use of an inducible IRES formed upon translation of the μORF. Both transcriptional and translational activation require the phosphorylation of the eukaryotic initiation factor 2α. The AARE and ARE are necessary for enhancement of transcription and mRNA stability, respectively. *See* text for more detail. (*B*) The 3′UTR of the hCAT-1 mRNA contains recognition sequences for the liver-specific microRNA miR-122. Release of miR-122-mediated translational repression occurs upon amino acid starvation or other cellular stress and requires binding of HuR to a region downstream of the miR-122 binding sites. This has been shown for the short hCAT-1 mRNA but is likely to be also true for the large message that contains an additional miR-122 binding site. (*C*) The 3′UTR of the large mCAT-2 message contains three 100 nt inverted repeats (*green*). These can form imperfect stem loops with a forward repeat (red) that undergoes A-to-I editing and is located in a region common to both, the short and the long message. Cleavage of the long message is supposed to be necessary for nuclear export and translation.

occurs much more rapidly than in C6 cells, does not require phosphorylation of eIF2α and is inhibited by cycloheximide. We are thus looking at two different types of translational activation: in C6 cells, a low-efficiency basal translation takes place in the fed (unstressed) condition and is turned into highly efficient translation under stress conditions. In contrast, in Huh7 cells, basal translation is suppressed by miR-122 and stress-induced release of this suppression leads to a basal translation rate probably at a relatively low level similar to C6 cells in the unstressed condition. Whether the IRES-mediated translational enhancement also takes place in human cells needs investigation. Of the three alternative 5′UTRs found in hCAT-1 transcripts, two contain a μORF. One of them resembles in length and position the μORF in the 5′UTRs of rCAT-1 in C6 cells (Bhattacharyya et al., 2006). Interestingly, transcripts containing this 5′UTR are not present in unstressed Huh7 cells, but appear after 3 h of starvation.

The miR-122-mediated specific downregulation of CAT-1 in hepatocytes most likely serves to protect plasma arginine from hydrolysis by arginase, which is highly expressed in these cells. Rapid release of this repression under certain conditions is probably necessary to sustain vital cellular functions such as hepatocellular protein synthesis. However, under complete amino acid depletion, no extracellular substrate is available for the newly synthesized transporter to pump into the cell. So why is it made? One hypothesis implies that a transporter reserve is installed to enable the starved cell to quickly replenish the intracellular amino acid pool, once substrate is again available. This process has been referred to as adaptive de-repression and was first observed for the Na^+-dependent, neutral amino acid transport system A (Gazzola et al., 1972). In contrast to CAT-1, induced in C6 cells by deprivation of any given amino acid or even of glucose (Fernandez et al., 2002a), regulation of system A occurs only in response to substrate amino acids. In contrast to system A concentrative transporter, CAT-1 also mediates substrate efflux and thus potentially contributes to a further loss of its substrates, e.g., at low membrane potential. However, in the case of glial cells, efflux of amino acids under amino acid starvation may serve to provide neurons with essential nutrients. Investigation of the adaptive de-repression in other cell types will show whether this process is regulated in a cell-specific manner.

So far the translational control of CAT-1 has been studied under complete deprivation of single or all amino acids and it is not known at which level of deprivation these mechanisms take effect and if such low levels ever occur in vivo. Plasma amino acid concentrations are pretty stable even over longer periods of starvation, making an overall depletion unlikely. However, drastic changes may occur locally, e.g., when amino acid-consuming enzymes, such as arginase or indoleamine 2,3-dioxygenase (IDO, a tryptophan-metabolizing enzyme) are induced (Morris, 2006; Muller & Prendergast, 2005). Exchange transporters

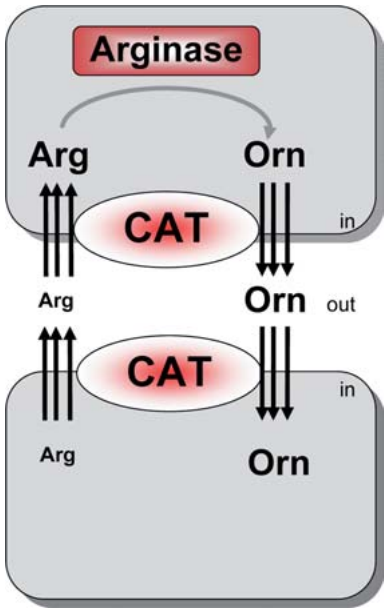

Fig. 3. Involvement of CAT proteins in arginine signaling. A cell with high arginase activity (*top*) depletes the extracellular medium of arginine by high arginine consumption. In addition, through exchange of ornithine for arginine, neighboring cells (*bottom*) will be depleted of arginine. It is proposed that the CAT-mediated amino acid exchange is necessary for efficient depletion and to trigger consequential changes in the neighboring cell.

such as the CAT proteins may have an important function in the signaling pathway of these enzymes, because they do not only deliver substrate for the enzymatic reaction but also cause efficient depletion of the neighboring cells by substrate/product exchange (arginine/ornithine in the case of arginase, *see* Fig. 3). Thus, an alternative explanation for the unopposed activation of DC in CAT-2 knockout mice (Rothenberg et al., 2006), discussed in the previous section, may be a lack of DC arginine deprivation by macrophage arginase.

A complex mechanism of translational control has also been proposed for CAT-2. Prasanth and coauthors observed nuclear retention of the large (about 8 kb) mCAT-2 transcript in the nucleus of C127I mouse mammary tumor cells (Prasanth et al., 2005). They also demonstrated that the mCAT-2 mRNA localizes to paraspeckles, nuclear subdomains associated with splicing speckles, and associates with specific paraspeckle proteins in NIH 3T3 fibroblasts and mouse embryonic fibroblasts. Using reporter constructs, they show that the 3′UTR of the long mCAT-2 transcript, e.g., a 100 nt fragment that undergoes A-to-I editing, is responsible for the nuclear retention and leads to reduced translation of reporter proteins. Under transcriptional inhibition, the 8 kb mRNA is degraded to a smaller RNA. They conclude that the large mCAT-2 transcript is stored away in the nucleus and must be cleaved for nuclear export and translation. The pitfall with this study is, however, that endogenous mCAT-2 protein expression has not been investigated. In the numerous reports showing Northern blots of CAT-2, the ratio between long and short CAT-2 messages consistently seems to be similar (the large message being the predominant species, irrespective of the splice variant expressed) and there is no hint that a change in this ratio must occur for protein synthesis. Although Prasanth and colleagues discuss how CAT-2B induction under inflammation and "stress" might lead to the appearance of the shorter mRNA species, the nuclear retained RNA they published is CAT-2A, which is constitutively expressed at both RNA and protein level in liver and pancreas, in the absence of stress (Closs et al., 1993b). An alternative explanation would be that the mCAT-2 3′UTR retains the mRNA in paraspeckles until alternative splicing has occurred. Reporter constructs without alternative exons may thus be retained in the nucleus. It would be interesting to see in this context, if the mCAT-2 message retained in the nucleus of the particular cell line (C127I) has a splicing defect.

Subcellular Localization of the CAT Proteins

Conflicting results have been published regarding the subcellular localization of CAT-1. In porcine aortic endothelial cells (PAEC), human fibroblasts and rat hepatoma cells, endogenous CAT-1 was found to be soluble in triton and localized in clusters (not resembling caveoli) that were dispersed by disrupting microtubules (Woodard et al., 1994). In contrast, a later study reported CAT-1 to be associated with endothelial NOS (eNOS) and caveolin in PAECs (McDonald et al., 1997). The localization of overexpressed CAT-1 (tagged with green fluorescent protein, GFP) has been studied in different cell lines. A predominant localization of CAT-1-GFP in caveoli has been reported in baby hamster kidney cells (BHKs) (Lu & Silver, 2000). In contrast, the majority of CAT-1-GFP is found in intracellular vesicles in U373 human glioblastoma cells, and only a small part in the plasma membrane, where it localizes mainly to filopodia and ruffled borders (Rotmann et al., 2004b; Wolf et al., 2002). In polarized Madin Darby canine kidney (MDCK) and HEK 293 cells, CAT-1-GFP is confined to the basolateral membrane (Cariappa et al., 2002; Kizhatil & Albritton, 2002). However, in nonconfluent HEK cells, CAT-1 is mostly detected in filopodia and the golgi apparatus (Masuda et al., 1999). Deletion of amino acids 4 through 33 leads to retention of the majority of CAT-1 in the ER in BHK cells, but not in HEK 293 cells (Ou & Silver, 2003). Taken together, the subcellular localization of CAT-1 seems to be regulated in a cell-specific manner. In U373 cells GFP-tagged CAT-3 and SLC7A4 also

exhibit strong intracellular accumulation, similar to that seen for CAT-1 (Wolf et al., 2002; Rotmann et al., 2006). However, this may be different in neurons or other cell types that express these isoforms endogenously (Hosokawa et al., 1999).

Activation of protein kinase C (PKC), most likely PKCα, in mammalian cells or *Xenopus laevis* oocytes leads to a decrease in the cell surface expression of CAT-1 and CAT-3 (Rotmann et al., 2004b, 2006). This explains the reduced transport activity observed upon PKC activation in different cell types (Gräf, Förstermann & Closs, 2001; Krotova, Zharikov & Block, 2003; Zharikov et al., 2004). The PKC effect on the subcellular localization of hCAT-1 seems to be reversible, as arginine transport recovers quickly after removal of the PKC activation agent (Rotmann et al., 2004b). In addition, total protein content as well as protein turnover are not affected by PKC. hCAT-1 does not seem to be phosphorylated by PKC either directly or indirectly, indicating the involvement of intermediate proteins. So far it is not clear if the reduced CAT expression at the cell surface is due to increased internalization or decreased delivery of the transporter to the plasma membrane. Internalization of mCAT-1 occurs upon binding of envelope protein from ecotropic murine leukemia virus and does not involve dynamin or clathrin-coated pits (Lee, Zhao & Anderson, 1999). It would be interesting to find out if this process is PKC-dependent. A stimulation of system y^+ activity by PKC has also been reported (for review *see* Closs, 2002). With one exception (Racke et al., 1998) the latter occurs only after several hours incubation with the PKC-stimulating phorbol ester phorbol-12-myristate-13-acetate (PMA) and might therefore be due to PMA-induced increase in CAT-1 expression. Changes in the subcellular localization of the CAT proteins may represent an important regulatory mechanism in addition to the expressional regulation discussed above. In this context, it would be interesting to determine the subcellular localization of CAT-1 proteins following amino acid starvation.

Regulation of CAT Transport Activity

For system y^+-(CAT-1)-transporter localized in the plasma membrane, there are two major determinants of transport activity: trans-stimulation and membrane potential. The intracellular concentration of cationic amino acids is thus an important determinant for the rate of initial radiotracer uptake. In cells with initially low concentrations of intracellular cationic amino acids, a rise in the level of these amino acids (e.g., by protein breakdown) may result in an increase of CAT-1-mediated substrate uptake. In addition, net substrate transport by CAT-1 is voltage-dependent, with hyperpolarization increasing influx and decreasing efflux rates (Kavanaugh, 1993; Rotmann et al., 2004a). This is consistent with earlier reports demonstrating the voltage dependence of net flux through system y^+ (Bussolati et al., 1987, 1989). Membrane hyperpolarization may also explain why arginine influx increases in endothelial cells after application of bradykinin (Bogle et al., 1996), adenosine (Sobrevia, Yudilevich & Mann, 1997), insulin (Gonzalez et al., 2004) or glucose (Flores et al., 2003), and in endothelial cells from diabetic patients (Sobrevia et al., 1995). The other CAT isoforms depend less on trans-stimulation and under physiological conditions also on membrane potential. They may thus allow efficient influx of cationic amino acids into depolarized and substrate-depleted cells. This may, for instance, explain why CAT-2B is necessary for sustained substrate supply to iNOS in activated macrophages, as these cells exhibit a low membrane potential and have a high consumption of cationic amino acids.

Zharikov and co-authors reported that interaction with cytoskeletal proteins modulates CAT-1 activity without affecting the CAT-1 protein content (Zharikov & Block, 2000; Zharikov et al., 2001). Down-regulation of system y^+ (CAT) activity in response to short-term treatment with platelet-derived growth factor (PDGF) (Durante et al., 1996), lysophosphatidylcholine (LPC) (Durante et al., 1997), thrombin (Durante et al., 1998) and transforming growth factor (TGF-β) (Durante et al., 2001) has been reported in vascular smooth muscle cells (SMC), and for LPC also in endothelial cells (Kikuta et al., 1998). It remains, however, to be elucidated whether the reduced transport rate in the latter cases is due to a decrease in CAT-1 expression, a change in subcellular localization, altered transport activity or a reduction in the apparent substrate affinity.

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