Increased Cat3-mediated Cationic Amino Acid Transport Functionally Compensates in Cat1 Knockout Cell Lines*

(Received for publication, April 9, 1998, and in revised form, April 21, 1998)

Benjamin Nicholson‡§, Tatsuya Sawamura%, Tomoh Masaki, and Carol L. MacLeod¶

From the §San Diego Cancer Center and Department of Medicine, University of California, La Jolla, California 92037-0684 and ¶Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto 606, Japan

Arginine transport is important for a number of biological processes in vertebrates, and its transport may be rate-limiting for the production of nitric oxide. The majority of L-Arg transport is mediated by System y⁺, although several other carriers have been kinetically defined. System y⁺ cationic amino acid transport is mediated by proteins encoded by a family of genes, Cat1, Cat2, and Cat3. High affinity L-arginine transport was investigated in embryonic fibroblast cells derived from Cat1 knockout mice that lack functional Cat1. Both wild type and knockout cells transport arginine with comparable $K_m$ and $V_{max}$. However, the apparent affinity for lysine transport was 2.4 times lower in Cat1⁻/⁻ cells when compared with wild type cells, a property characteristic of Cat3-mediated transport. Northern analysis documented Cat2 mRNA increased 2-fold, whereas Cat3 mRNA levels increased 11-fold in Cat1⁻/⁻ relative to Cat1⁺/+ cells. The low affinity Cat2a mRNA was not detectably expressed in these cells. Even though Cat3 expression is normally limited to adult brain, there was a large increase in the amount of Cat3 protein present at the plasma membrane of Cat1⁻/⁻ embryonic fibroblast cells. These results suggest that Cat3 compensates for the loss of functional Cat1 in cells derived from Cat1 knockout mice and mediates the majority of high affinity arginine transport.

Since first kinetically characterized by Christensen in the 1960s, L-Arg uptake has been found to be mediated by a number of competing systems such as System y⁺ (1–3), b⁺/y⁺ (4), and y⁺L (5) (for reviews see Refs. 6–9). System y⁺ is a sodium-independent transporter with a high affinity for the l-isomers of cationic amino acids and is subject to trans-stimulation (2). System y⁺ is widely believed to be the major carrier of cationic amino acids in adult tissues (6).

Three high affinity cationic amino acid transporters, Cat1, Cat2, and Cat3, were cloned serendipitously in 1989 (10), 1990 (11), and 1997 (12), respectively. Whereas these transporters share only about 61% amino acid sequence identity they were found to be kinetically indistinguishable with very similar $K_m$ values for l-arginine in oocytes (13, 14). Cat1 appears to have a greater capacity than Cat2 for trans-stimulation (14), whereas an alternately spliced transcript of Cat2 encodes a kinetically distinct low affinity form of Cat2a (15). Cat3 was cloned from mouse (12) and rat (16). In both species, Cat3 expression was localized to the brain although it was widely expressed in the 13.5-day postcoital mouse embryo. Unlike Cat1 or Cat2, Cat3 has different $K_m$ values for L-arginine (40–60 μM) and L-lysine (115–165 μM) uptake in Xenopus oocytes (12) and COS7 cells (1-Arg, 103 ± 12 μM; L-Lys, 147 ± 22 μM) (16). Hence, Cat3 can be kinetically distinguished from Cat1, Cat2, and Cat2a by their differential affinity to L-lysine. Similarly, a difference in the $K_m$ values for L-Arg and L-Lys transport in HTC hepatoma cells was previously reported (3) but never assigned to a particular Cat family member. The importance of Cat1-mediated transport was recently underscored by the production of knockout mice (17). Mice homozygous for this mutation die within 12 h of birth; they are severely anemic and runted. The knockout embryos express a truncated, non-functional Cat1 mRNA (17). Despite the apparent functional similarity of the Cat transporters, Cat1 function is essential for survival post parturition.

As with the glutamate transporters (for review, see Ref. 18) the Cat gene family appears to encode functionally redundant arginine transporter proteins (19). It is impossible to kinetically distinguish the contribution of one member of the Cat family by assessing arginine transport in the presence of competing members. Taking advantage of the differential affinity for lysine and a cell line established from the Cat1⁻/⁻ embryonic fibroblasts, we investigated arginine and lysine uptake into Cat1⁻/⁻ MEF cells. Results described in this paper will reveal that the ablation of Cat1 appears to be mainly compensated for by an enhanced expression of Cat3.

EXPERIMENTAL PROCEDURES

Materials—l-[2,3,4,5-3H]Arginine monohydrochloride, l-[4,5-3H]lysine monohydrochloride, [α-32P]dCTP, and the multiprime DNA labeling system were all from Amersham Pharmacia Biotech. Tissue culture reagents and Trizol were purchased from Life Technologies, Inc. Magnacharge nylon membranes were obtained from Micron Separations Inc. Titermax was from CytRx Co., and the rhodamine-conjugated goat anti-rabbit IgG was purchased from Jackson Laboratories Inc.

Both the Cat1⁻/⁻ and Cat1⁺/+ mouse embryonic fibroblasts were cultured in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin sulfate, and 10 μg/ml gentamicin sulfate. Four days prior to transport studies or RNA extraction, similar numbers of cells were plated into 35-mm tissue culture dishes and had reached confluence at the time of the experiments.

1 The abbreviations used are: Cat, cationic amino acid transporter; MEF, mouse embryonic fibroblast; gapdh, glyceraldehyde-3-phosphate dehydrogenase.
Transport Measurements—To avoid the effects of trans-stimulation (2) cells were washed once in HEPES buffer (140 mM choline chloride, 5 mM KCl, 0.9 mM CaCl₂, 1 mM MgSO₄, 5.6 mM d-glucose, and 25 mM HEPES, pH 7.4) and incubated in HEPES buffer for 2 h at 37 °C. Na⁺-independent transport experiments were performed as described (21) with the following modifications. First, cells were washed (4 × 1.25 ml) with transport buffer (137 mM choline chloride, 5.4 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 10 mM HEPES, adjusted to pH 7.4 with Tris). Amino acid transport was instigated by the addition of 1 ml of transport buffer supplemented with L-arginine and 5 mM L-Leu. 38 nm L-[2,3,4,5-³H]arginine monohydrochloride was mixed to a final concentration of 250 μM L-Arg (unless stated otherwise) with unlabeled L-Arg in the supplemented transport buffer. Competition analysis revealed significant L- and b⁰⁺ activity in the embryonic fibroblasts that was inhibited with 5 mM L-Leu. Unless stated otherwise all transport experiments were performed over 3 min at 24 °C. The transport was terminated by rapidly washing (4 × 1.25 ml) with ice-cold 137 mM NaCl, 10 mM Tris, 10 mM HEPES, pH 7.4. Non-saturable binding plus uptake was measured in parallel in the presence of 38 nm L-[2,3,4,5-³H]arginine, 5 mM L-Arg, and 5 mM L-Leu. Above 3 mM L-Arg, no significant saturable uptake of L-Arg occurred. Therefore, the disintegrations per min (dpm) in the cells following washing represented the non-saturable and binding components of arginine uptake into mouse embryonic fibroblasts. The dpm at 5 mM were normalized for the protein content and subtracted from the dpm/mg at lower arginine concentrations to reveal the saturable uptake of L-arginine into the mouse embryonic fibroblasts (22). L-Lysine transport studies were identical except ³H-labeled L-Arg and unlabeled L-Arg were replaced with L-[4,5-³H]lysine monochloroiodide and unlabeled L-Lys, respectively. In experiments measuring trans-stimulation of transport, cells were pre-incubated with 10 mM L-arginine for 2 h in HEPES buffer as described above.

RNA Extraction and Northern Blot Analysis—RNA was isolated from cells previously washed and cultured for 2 h in HEPES buffer using Trizol according to the manufacturer's instructions. 10 μg of total cellular RNA was resolved on a 0.8% (w/v) agarose gel under denaturing conditions, transferred overnight to Magnacharge blotting membrane in 20× SSC (0.15 M NaCl, 15 mM sodium citrate, pH 7.0), and cross-linked by baking for 2 h at 80 °C. Blots were prehybridized at 42 °C in 50% (v/v) formamide, 10% (w/v) dextran sulfate, 5× standard sodium phosphate-EDTA (0.6 M NaCl, 40 mM NaH₂PO₄, 4 mM EDTA, pH 7.4), and 5× Denhardt's solution (1 g/liter bovine serum albumin, 1 g/liter Ficoll). 0.1 mg/ml denatured sonicated salmon sperm DNA, and 0.5% (w/v) SDS. 10⁶ cpm/ml of individual cDNA probes labeled with [³²P]dCTP using the multiprime DNA labeling system were hybridized to blots overnight at 42 °C. Blots were washed twice for 20 min at 42 °C with 1× SSC, 0.1% (w/v) SDS and once at 42 °C with 0.1× SSC, 0.1% (w/v) SDS before exposure to PhosphorImager cassettes. Densitometry was performed using a PhosphorImager (Molecular Dynamics). When required the blot was stripped with 0.1% (w/v) SDS at 100 °C.

A

PROCEDURE FIG. 1. System y⁺ uptake of L-arginine in Cat1−/− mouse embryonic fibroblasts. To achieve zero trans-stimulation the cells were washed once with HEPES buffer and subsequently incubated for 2 h at 37 °C in HEPES buffer. The uptake of 250 μM L-Arg was determined in the presence or absence of competing amino acids in the transport buffer (A). In all cases except L-glutamine (10 mM) and L-aspartate (10 mM) the competing amino acid was present at 20 mM. With the exception of L-His (pH 7.0) the uptake was performed at pH 7.4. The data are presented as a percentage of the control saturable arginine uptake and are means ± S.E. of triplicate determinations. The saturable and non-saturable uptake of L-arginine was determined over the time course shown (B). Saturable uptake was calculated by subtracting the non-saturable uptake of L-Arg from the total uptake of L-Arg at each time point. The inset shows the saturable and non-saturable uptake of L-arginine over the first 5 min. The results presented are the mean ± S.E. of three determinations in each case. Where not indicated the error bars appear within the data points. *, p < 0.05 versus rate in the absence of competing amino acids; **, p < 0.01 versus rate in the absence of competing amino acids; ***, p < 0.001 versus rate in the absence of competing amino acids.

B

Generation of Cat3-specific Antisera—A synthetic peptide corresponding to amino acids 218–238 of mouse Cat3 (12, 16) was synthesized using mitogen-activated protein resin as a solid phase. One mg of the peptide was emulsified with 0.2 ml of Titermax and injected intradermally in the interscapulovertebral region of New Zealand White rabbits. Four immunizations were carried out at 2-week intervals. The sera were collected by centrifugation and stored at −20 °C.

Immunofluorescence—Embryonic fibroblasts were cultured on poly-d-lysine-coated coverslips. Cat3 protein expression was assessed by immunofluorescence essentially as described (23). Cat3 antisera was diluted 1:40 for use in these assays. The anti-rabbit-rhodamine conjugate was used at 1:200. The slides were visualized on an Axioptop epifluorescence microscope.

RESULTS AND DISCUSSION System y⁺ Transport Activity in Cat1−/− and Cat1+/+ MEF Cells—Cationic amino acid transport was evaluated in Cat1−/−embryonic fibroblasts by measuring the initial rate of 250 μM L-Arg uptake in the presence and absence of other amino acids (Fig. 1A). Two substrates of System y⁺, L-Lys and L-ornithine, both inhibited L-arginine uptake by greater than 90% consistent with System y⁺ activity (2). L-Arginine uptake was also measurably inhibited by a weaker System y⁺ substrate, L-His (24), that is partially cationic at pH 7.0 (13, 25). As expected, the neutral (L-alanine and L-glutamine) and acidic (L-aspartate) amino acids had no effect on L-arginine uptake. Interestingly, the capacity of v-arginine to partially inhibit L-Arg uptake into Cat1−/− cells is characteristic of Cat3 transport (12, 16). These data indicate that both Cat1−/− and Cat1+/− MEF cells possess System y⁺ transport activity.

Saturable 250 μM L-arginine uptake approached a steady value after 30 min, and the amount of arginine accumulated by cells increased almost linearly with time for 5 min (Fig. 1B). Hence, transport experiments were measured over 3-min intervals. Although System y⁺ transporters are capable of cationic amino acid efflux, the linearity of arginine uptake indicates little L-Arg export from the cells over the first 5 min. In addition, it had been shown previously in human fibroblasts that over 40 min there was measurable metabolism of L-Arg (2). At concentrations above 3 mM L-arginine, the contribution of saturable transport to the overall accumulation of arginine is not significant (data not shown). Therefore, by measuring the uptake of tracer amounts of ³H-labeled L-arginine in the presence of 5 mM L-arginine it was possible to determine the non-saturable component of L-arginine uptake (22). As expected, the non-saturable uptake of L-Arg did not reach a steady value.
over the time of the experiment. There were two distinct components to the non-saturable uptake, an initial rapid uptake that is complete within the first min and a slower uptake over the next 30 min (Fig. 1B, inset). The two non-saturable components could correspond to a rapid nonspecific binding of the cationic amino acid to the negatively charged plasma membrane and a slower diffusion component.

The different affinities of Cat2 and Cat3 for L-lysine were exploited to identify the protein(s) responsible for System y\(^+\) transport activity in Cat1\(^{-/-}\) MEF cells (Fig. 2, Table I). The \(K_m\) of L-lysine uptake into Cat1\(^{-/-}\) MEF cells was 2.4-fold higher than the \(K_m\) of L-lysine transport into Cat1\(^{+/+}\) cells. In contrast, the \(K_m\) of L-arginine uptake into Cat1\(^{-/-}\) and Cat1\(^{+/+}\) cells was not significantly different. Cat3 is the only member of the gene family known to transport L-Lys with a lower affinity than L-Arg; hence this provides kinetic evidence that Cat3 is responsible for System y\(^+\) transport activity in Cat1\(^{-/-}\) MEF cells. The \(V_{\text{max}}\) values for L-Arg or L-Lys uptake were comparable.

Fig. 2. Kinetic analysis of cationic amino acid transport into Cat1\(^{-/-}\) and Cat1\(^{+/+}\) MEF cells. The \(K_m\) and \(V_{\text{max}}\) of saturable L-Arg (A, B) and L-Lys (C, D) transport into Cat1\(^{-/-}\) (A, C) and Cat1\(^{+/+}\) (B, D) MEF cells was determined by Eadie-Hofstee plots. The initial rate of uptake (c) is presented as nmol/mg/min. The lysine or arginine concentration is \(\mu\text{M}\). Means ± S.E. of triplicate determinations are shown. Where not indicated the error bars appear within the data points.

Table I

| Amino acid | Genotype | \(K_m\) \(\mu\text{M} \pm \text{S.E.}\) | \(V_{\text{max}}\) \(\text{nmol/mg/min} \pm \text{S.E.}\) | Trans-stimulation |
|------------|-----------|-------------------------------|---------------------------------|------------------|
| L-Arginine | Cat1\(^{+/+}\) | 107.1 ± 4.1 | 0.42 ± 0.01 | 4.9 ± 0.1\(^a\) |
|           | Cat1\(^{-/-}\) | 93.7 ± 8.1 | 0.41 ± 0.01 | 2.3 ± 0.2\(^b\) |
| L-Lysine  | Cat1\(^{+/+}\) | 95.0 ± 4.8 | 0.39 ± 0.01 | ND\(^c\) |
|           | Cat1\(^{-/-}\) | 228.7 ± 21.2 | 0.36 ± 0.02 | ND\(^c\) |

\(^a\) \(p < 0.001\) versus \(K_m\) of L-lysine uptake into Cat1\(^{-/-}\) MEF cells.

\(^b\) \(p < 0.001\) versus initial rate of L-Arg uptake in zero trans-stimulated cells.

\(^c\) ND, not determined.

To differing degrees, all three high affinity Cat transporters are capable of trans-stimulation (Cat1 \(>\) Cat2 \(=\) Cat3). Table I shows System y\(^+\) trans-stimulation in both the Cat1\(^{+/+}\) and Cat1\(^{-/-}\) MEF cells following preloading of the cells with 10 mM L-Arg (Table I). The initial rate of L-Arg transport increased 4.9 ± 0.1-fold in the Cat1\(^{-/-}\) fibroblasts following trans-stimulation. As Cat1 is the only member of the Cat family reported to be trans-stimulated by greater than 3-fold (14) it is likely that Cat1 is responsible for the bulk of the observed trans-stimulation. The 2.3 ± 0.2-fold increase in the initial rate of L-arginine transport in Cat1\(^{-/-}\) cells following trans-stimulation is in agreement with the reported values for Cat2 (14) and Cat3 (12). Furthermore, the finding that trans-stimulation in Cat1\(^{-/-}\) cells is less than 3-fold supports the conclusion that no functional Cat1 transporter is present in Cat1\(^{-/-}\) cells (17).

Cat3 is induced in Cat1\(^{-/-}\) MEF Cells—The relative steady state mRNA expression levels of all three Cat high affinity transporters were assessed in Cat1\(^{-/-}\) and Cat1\(^{+/+}\) cells by Northern analysis (Fig. 3, A and B). Although a small amount of Cat1 RNA was observed in the Cat1\(^{-/-}\) cells, it is non-functional (17). Fig. 3B shows Cat3 mRNA was increased 11.1 ± 0.5-fold and Cat2 mRNA 2.0 ± 0.3-fold in Cat1\(^{-/-}\) cells as assessed by PhosphorImager analysis (Fig. 3B). No detectable low affinity transporter Cat2a mRNA was detectable in either cell type (data not shown). It is valid to relate the expression of individual Cats on Northern blots between the two genotypes, but a direct comparison of the mRNA levels does not necessarily reflect the amount or activity of the transport proteins themselves. Nevertheless, the data presented in Fig. 3, A and B provide a good indication of the steady state mRNA levels of the high affinity arginine transporters. Hence, at the mRNA level it appears that Cat3 compensates for the ablation of Cat1.

To confirm this observation, immunofluorescence was performed using a specific Cat3 antibody. Fig. 3C shows a substantial increase in Cat3 protein expression in the Cat1\(^{-/-}\) cell line. This conclusion is supported by the transport characteristics of the cells and the observed changes in Cat gene expression. Analysis of Cat1\(^{-/-}\) and Cat1\(^{+/+}\) cells revealed that the knockout cells (i) have a higher apparent \(K_m\) for L-lysine transport, (ii) have significantly increased Cat3 mRNA, (iii) show increased Cat3 immunoreactivity, and (iv) transport L-arginine with indistinguishable kinetics. However, Cat2 mRNA is also increased in the Cat1\(^{-/-}\) cells (Fig. 3B) and could contribute to the functional compensation conferred by Cat3. To precisely determine the proportion of cationic amino acid transport attributable to Cat2 and/or Cat3 would require their selective inactivation and would be informative only if no currently unidentified Cat family members were expressed in
Cat3 Compensates for Cat1 in Cat1−/− Cells

These cells. Hence, it is not yet possible to precisely resolve the contribution of Cat2 and Cat3 to cationic amino acid transport. Because the large increase in Cat3 expression failed to detectably alter the $V_{\text{max}}$ of arginine or lysine transport it is likely that an equivalent amount of Cat3 protein substitutes for the loss of Cat1. Alternatively, Cat3 protein may be present in larger amounts than Cat1 in wild type cells, but transport may be limited by an unknown cellular factor, as suggested for the high affinity glutamate transporter, EAA1 (26). Cat2 gene expression in adult mice is limited to a few cell types and tissues and is induced in circumstances of high arginine demand (reviewed in Ref. 19), whereas Cat3 expression appears to be limited to the brain of adult mice but is reported to be widely expressed during embryogenesis (12). Although Cat3 expression may functionally compensate for the ablation of Cat1 in Cat1−/− MEF cells, further investigation is required to determine whether alteration of either or both Cat3 or Cat2 expression in Cat1−/− mice explains embryonic survival until birth.

Acknowledgments—We are grateful to Dr. Chris Perkins for the Cat1−/− cell line and to Drs. Lon Van Winkle and John McGivan for careful guidance and helpful advice in designing the transport experiments.

REFERENCES

1. Christensen, H. N., and Antonioli, J. A. (1969) J. Biol. Chem. 244, 1497–1504
2. White, M. F., Gazzola, G. C., and Christensen, H. N. (1982) J. Biol. Chem. 257, 4443–4449
3. White, M. F., and Christensen, H. N. (1982) J. Biol. Chem. 257, 4450–4457
4. Van Winkle, L. J., Camponio, A. L., and Gorman, J. M. (1988) J. Biol. Chem. 263, 3150–3163
5. Deves, R., Chavez, P., and Boyd, C. A. R. (1992) J. Physiol. (Lond.) 454, 491–501
6. Malandro, M. S., and Kilberg, M. S. (1996) Annu. Rev. Biochem. 65, 305–336
7. MacLeod, C. L., and Kakuda, D. K. (1996) Amino Acids 11, 171–191
8. Closs, E. I. (1996) Amino Acids 11, 193–208
9. Palacín, M., Mora, C., Chillaritín, J., Calonge, M. J., Estévez, R., Torres, D., Testar, X., Zorzano, A., Nunes, V., Parroy, J., Estivill, X., Gasparini, P., Biseeglia, L., and Zelante, L. (1996) Amino Acids 11, 225–246
10. Albritton, L. M., Tseng, L., Scadden, D., and Cunningham, J. M. (1989) Cell 57, 659–666
11. MacLeod, C. L., Finley, K., Kakuda, D., Kozak, C., and Wilkinson, M. (1990) Mol. Cell. Biol. 10, 3683–3674
12. Ito, K., and Groudine, M. (1997) J. Biol. Chem. 272, 26780–26786
13. Kakuda, D. K., Finley, K., Dionne, V. E., and MacLeod, C. L. (1995) Transgene 1, 91–101
14. Closs, E. I., Graf, P., Habermeier, A., Cunningham, J. M., and Förstermann, U. (1997) Biochemistry 36, 6462–6468
15. Closs, E. I., Albritton, L. M., Kim, J. W., and Cunningham, J. M. (1993) J. Biol. Chem. 268, 7538–7544
16. Hosokawa, H., Sawamura, T., Kobayashi, S., Ninomiya, H., Miwa, S., and Masaki, T. (1997) J. Biol. Chem. 272, 8717–8722
17. Perkins, C. P., Mar, V., Shutter, J. R., del Castillo, J., Danilenko, D. M., Medlock, E. S., Ponting, I. L., Graham, M., Stark, R. L., Zuo, Y., Cunningham, J. M., and Besselman, R. A. (1997) Genes Dev. 11, 914–925
18. Kanai, Y. (1997) Curr. Opin. Cell Biol. 9, 565–572
19. MacLeod, C. L. (1996) Biochem. Soc. Trans. 24, 846–852
20. Pink, D., Zheng, H., Nobe, S., Norris, P. S., Aebi, S., Lin, T-P., Nehme, N., Christen, R. D., Haas, M., MacLeod, C. L., and Howell, S. B. (1997) Cancer Res. 57, 1841–1845
21. Plakidou-Dymock, S., and McGivan, J. D. (1993) Biochem. J. 295, 749–755
22. Durante, W., Liao, L., and Schaefer, A. I. (1995) Am. J. Physiol. 268, H1158–H1164
23. Woodard, M. H., Dunn, W. A., Laine, R. O., Malandro, M., McMahon, R., Sinell, O., Block, E. R., and Kilberg, M. S. (1994) Am. J. Physiol. 266, E817–E824
24. Wang, H., Kavanaugh, M. P., North, R. A., and Kabat, D. (1991) Nature 352, 729–731
25. Kim, J. W., Closs, E. I., Albritton, L. M., and Cunningham, J. M. (1991) Nature 352, 725–728
26. Nicholson, B., and McGivan, J. D. (1996) J. Biol. Chem. 271, 12159–12164