Characterization of the Third Member of the MCAT Family of Cationic Amino Acid Transporters

IDENTIFICATION OF A DOMAIN THAT DETERMINES THE TRANSPORT PROPERTIES OF THE MCAT PROTEINS*

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Ellen Idichio Closs, C. Richard Lyons, Carol Kelly, and James M. Cunningham

From the Howard Hughes Medical Institute and Division of Hematology/Oncology, Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02115

We have identified the third member of a family of cationic amino acid transporters in lipopolysaccharide-stimulated murine macrophages. The deduced amino acid sequence of this transporter is the same as MCAT-2 (mouse cationic amino acid transporter-2), the low affinity transporter expressed in hepatocytes, except for a stretch of 41 amino acids that connect the eighth and ninth membrane-spanning domains. These transporters apparently result from differential splicing of transcripts from a single gene and therefore have been named MCAT-2A (hepatocyte) and MCAT-2B (macrophage). Despite their similarity, MCAT-2B is saturated at one-fifth the arginine concentration, has a lower apparent $V_{\text{max}}$ and is more sensitive to trans-stimulation than MCAT-2A. Introduction of the unique regions of MCAT-2A and MCAT-2B into the equivalent portion of the related protein, MCAT-1, created chimeric transporters with properties most like the donor of this region. Our findings suggest these 41 amino acids contain a domain that binds the amino acid substrate during its translocation across the membrane.

Previously, we identified two related transporters of the cationic amino acids, arginine, lysine, and ornithine (1, 2). They are members of a large family of distantly related proteins that include transporters of amino acids, choline and polyamines (3). MCAT-1 (mouse cationic amino acid transporter-1),\(^1\) has a high substrate affinity and is widely expressed in mouse tissues. In addition, its transport activity is increased by substrate on the opposite, or trans, side of the membrane. These properties are consistent with $y^+$, the principal mechanism for cellular uptake of cationic amino acids originally described by White and Christensen (4, 5). MCAT-1 also has the interesting property that it is a receptor for murine leukemia viruses (6). MCAT-2 has the same specificity for cationic amino acids as MCAT-1, but a 10-fold greater $K_m$ (Michaelis-Menton constant or half-saturating substrate concentration), and it is much less sensitive to trans-stimulation (2). MCAT-2 is expressed in hepatocytes, and therefore, we have suggested that it mediates the uptake of cationic amino acids from the portal vein after a large protein meal.

A cDNA encoding a protein that is related to these transporters has been identified previously in murine lymphomas (7). Our previous analysis of the reported sequence of this protein, termed Tea ($T$ cell early activation), demonstrated that it differs from the sequence of MCAT-2 in a stretch of 41 residues located between the eighth and ninth membrane-spanning domains and suggested it may not contain the N-terminal 205 residues. In this report, we describe the characterization of a cDNA encoding the complete Tea protein obtained from lipopolysaccharide-treated macrophages. Expression in Xenopus oocytes demonstrates that Tea is also a transporter of cationic amino acids. Therefore, we now refer to Tea as MCAT-2B and have renamed the hepatocyte transporter MCAT-2A. Functional studies of chimeric transporters demonstrate that the region of MCAT proteins which is divergent between MCAT-2A and MCAT-2B determines their transport properties.

MATERIALS AND METHODS

Isolation and Sequencing of the MCAT-2B cDNA—Ten micrograms of RNA, prepared by guanidine isothiocyanate/CsCl procedure from RAW264.7 macrophages exposed to lipopolysaccharide (1 mg/ml) and interferon-$\gamma$ (50 units/ml) or from adult mouse liver served as templates to synthesize cDNAs using random hexamer primers and the SuperScript cDNA kit (Life Technologies, Inc.). Polymerase chain reactions (PCR, 40 cycles, annealing temperature 55°C) were performed on these cDNAs using the sense oligonucleotide, GGAAGATCTTGCTTTGTAAAAGTTTGA (including a BgII site, underlined, and bp 859–928 of p20.5, (7)), as the antisense primer. The amplification product obtained by reverse transcription/PCR from macrophage RNA was digested with BgII and subcloned into the BamHI site of pBSIKS+ (Stratagene, La Jolla, CA). The nucleotide sequence of two independent clones was determined by dideoxynucleotide chain termination using Sequenase 2.0 (United States Biochemical Corp.).

A cDNA containing the entire coding sequence of MCAT-2B was constructed by replacing the 370-bp PstI/KpnI restriction fragment of pHM324, the plasmid that contains the complete coding portion of MCAT-2A (2), with the 570-bp PstI/KpnI fragment from the Tea-encoding plasmid, p20.5, containing the stretch of nucleotides that differs in sequence from MCAT-2A. The 900-bp PstI fragment of pHM324 was then reinserted into the PstI site of this clone to create a plasmid containing the complete coding portion of MCAT-2B.

Construction of Chimeric cDNAs—A BamHI recognition site was created in the MCAT-2A cDNA by PCR using the sense primer, CTGG-GATCCATGATTTCCCTGCA (BamHI site, underlined, and bp 1079–1099 of MCAT-2A) and the antisense primer, GCATTGGTACATGATTCG (bp 1379–1359 of MCAT-2A). The amplified product was digested with BamHI and KpnI and substituted for the corresponding 240-bp

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\(2\) To whom correspondence should be addressed: Rm. 925, Thorn Bldg., Brigham and Women's Hospital, 75 Francis St., Boston, MA 02115.

\(1\) The abbreviations used are: MCAT, mouse cationic amino acid transporter; Tea, T cell early activation protein; PCR, polymerase chain reaction; bp, base pairs.)
Amino Acid Uptake—All cDNAs were cloned into the BgZII site of the closed BamHYKpnI fragment of MCAT-2B. Two DNA fragments were synthesized by in vitro transcription (transcription kit Stratagene, Pont-New England Nuclear) in 50 nl of water were prepared by PCR to create a BamHI recognition site at the corresponding position (bp 1244–1249) in the MCAT-1 cDNA. First, a portion of the MCAT-1 cDNA was amplified using the sense primer, GCGGATCCATGCCCATG (BamHI site underlined, and bp 1231–1261 of MCAT-1), and the antisense primer, GTTCCCCATG (BamHI site underlined, and bp 1231–1261 of MCAT-1). The second fragment was synthesized using the same template and the sense primer, CAGGATCCCATGTTGCCCATG (BamHI site underlined, and bp 1266–1297 of MCAT-1), and the antisense primer, GCGGATCCATTGCACTGGTCC (containing bp 2067–2052 of MCAT-1). After digesting the amplified product of the first PCR with PstI and BamHI and the second PCR with BamHI and KpnI, both fragments were cloned into the PstI/KpnI site of pBSIIKS' in a three part ligation. The 240 bp AurI/KpnI fragment of the resulting plasmid was substituted for the equivalent AurI/KpnI fragment of MCAT-1. The deduced amino acid sequence of the proteins encoded by the MCAT-2A and MCAT-1 cDNA were not altered by introduction of the BamHI recognition sequence. The nucleotide sequences of the portions of these clones obtained by PCR were determined to assure that no additional changes were introduced by amplification. Chimeric cDNAs were constructed by exchanging the 240-bp BamHI/KpnI fragment of MCAT-1 by the equivalent AurI/KpnI fragment of MCAT-2A or MCAT-2B (the resulting cDNAs were termed MCAT-1/2A and MCAT-1/2B, respectively) and by replacing the 240-bp BamHI/KpnI fragment of MCAT-2 by the equivalent BamHI/KpnI fragment of MCAT-1 (the resulting cDNA was termed MCAT-2/1).

Expression of cRNAs in Xenopus laevis Oocytes and Measurement of Amino Acid Uptake—All cDNAs were cloned into the BglIII site of plasmid pSP64T (8). The resulting plasmids were linearized and sense RNAs were prepared by in vitro transcription (transcription kit Stratagene, La Jolla, CA), using 0.25 mCi/nmol [3H]UTP. Twenty-five nanoliters of RNA (1 ng/nl) were injected into each Xenopus oocyte, and amino acid uptake was determined as described previously (1). For trans-estimation experiments, 6 nml of [1-14C]arginine (2.5 nCi/oocyte, Du Pont-New England Nuclear) in 50 nl of water were injected into groups of oocytes. Three oocytes were pooled and transferred immediately to an isotonic salt solution (100 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM Hepes, 5 mM Tris-HCl, pH 7.5) containing a defined concentration of unlabeled arginine (0–2 mM). After 1 h, the efflux of [14C]arginine into the incubation medium was determined.

RESULTS

cDNA prepared from lipopolysaccharide-activated RAW264.7 mouse macrophages that express Tea transcripts served as a template to amplify Tea cDNA by PCR. An 1150-bp product was obtained using an antisense primer complementary to the coding portion of the Tea cDNA that differs from MCAT-2 and a sense primer derived from the sequence of MCAT-2 that includes the codon for the initiator methionine residue. No amplified product was obtained when the Tea-specific antisense primer was replaced by a primer complementary to the portion of the MCAT-2 cDNA that differs from the Tea cDNA or when the template synthesis reaction lacked reverse transcriptase. Both MCAT-2 and Tea-specific amplification products were obtained by PCR using a cDNA template prepared from mouse liver.

The nucleotide sequence of the 1150-bp amplification product obtained using the macrophage-derived cDNA as template is identical to the corresponding region of the cDNA encoding MCAT-2, except for the 73 bp adjacent to the Tea-specific antisense primer which is identical to the divergent sequence found in the Tea encoding cDNA. This suggests that Tea is 205 residues longer than initially reported and has the same N terminus as MCAT-2. Since the nucleotide sequences of Tea and MCAT-2 are identical except for the single divergent region that contains the primer sequence, these proteins are likely to be encoded by mRNAs derived by differential splicing of primary transcripts from the same gene. Therefore, we now refer to the Tea protein as MCAT-2B to reflect its probable origin from the same gene as MCAT-2, which we renamed MCAT-2A.

To construct a cDNA clone containing the complete coding region of MCAT-2B, the MCAT-2B-specific portion of p205.5 was substituted for the equivalent sequence in pMH324, the plasmid encoding MCAT-2A. Sense RNA prepared from the resulting plasmid was injected into Xenopus oocytes (25 ng/oocyte), and 2 days later, the uptake of radiolabeled amino acids by these oocytes was measured. Oocytes injected with MCAT-2B sense RNA accumulated 8–10-fold more of the cationic amino acids, arginine, lysine, and ornithine than water-injected oocytes. Uptake of these amino acids was not diminished significantly by substitution of choline chloride for sodium chloride in the incubation medium. No significant increase in the uptake of serine, alanine, glutamine, glutamic acid, tryptophan, leucine, phenylalanine, proline, methionine, histidine, cysteine, or citrulline was observed in the oocytes injected with MCAT-2B RNA (data not shown).

To characterize the kinetics of the MCAT-2B-mediated transport, the uptake of arginine as a function of the extracellular concentration between 0.025 and 1 mM was determined in Xenopus oocytes (Fig. 1). [14C]Arginine accumulation was measured over 15 min after preliminary experiments demonstrated that arginine uptake by these oocytes was linear over 1 h. In three independent experiments, the calculated apparent half-saturating substrate concentration (Km) was between 0.25 and 0.38 mM, and the maximal velocity (Vmax) was between 1.1 and 3.4 nmol/oocyte/h.

Previously, we determined that the transport activity of MCAT-1, but not MCAT-2A, is strongly dependent on the substrate concentration on the opposite or trans-side of the membrane. This property, termed "trans-stimulation" was evaluated for MCAT-2B by direct comparison with the two related carriers. Oocytes were injected with cRNA encoding each carrier and 2 days later, re-injected with 10 nmol of [14C]Arginine. After the second injection, groups of three oocytes were transferred immediately into incubation medium containing 0–2 mM

arginine, and efflux into the bath was determined after 1 h. Initial experiments demonstrated an increase in the efflux rate as a function of the extracellular arginine concentration between 0 and 250 μM. No further increase in the transport activity was observed at arginine concentrations up to 2 mM (data not shown). The results of an experiment demonstrating the efflux rate for each carrier at extracellular arginine concentrations between 0 and 250 μM is shown in Table 1. MCAT-1-mediated arginine efflux increased 8.3-fold by addition of 250 μM arginine to the incubation medium. MCAT-2A-mediated efflux in the arginine-free medium was almost 5-fold greater than MCAT-1, but increased only 52% upon addition of 250 μM arginine. Both the absolute rate of MCAT-2B-mediated efflux in arginine-free medium and the increase in efflux upon addition of arginine were intermediate between MCAT-1 and MCAT-2A. These findings are summarized in a plot of the carrier-mediated efflux expressed as a percentage of the rate at a bath concentration of 250 μM (Fig. 2).

Our findings demonstrate that MCAT proteins mediate flux of cationic amino acids in both directions across the cell membrane. Assuming $V_{\text{max}}$ is the same in both directions and the extracellular substrate concentration is sufficient to saturate influx, net inward transport will occur until the intracellular concentration of substrate becomes high enough to saturate outward transport, thereby equalizing the rate of influx and efflux. If true, the steady state level of substrate achieved by oocytes under these conditions provides an indirect measure of the substrate affinity of the transporter on the intracellular face of the membrane. The accumulation of arginine was monitored over 6 h by oocytes injected with MCAT cRNAs and incubated in 10 mM [14C]arginine (Fig. 3). Oocytes that expressed MCAT-1 reached equilibrium after accumulating only 1 nmol of arginine over 30 min. In contrast, oocytes that expressed MCAT-2A required 6 h to reach equilibrium and accumulated 2.5 nmol of arginine. These findings indicate that the apparent affinity of MCAT-1 for arginine on the intracellular face of the oocyte membrane is 10-fold greater than MCAT-2A and 2-fold greater than MCAT-2B.

These studies identify striking differences in the transport properties of MCAT-2A and MCAT-2B that must be determined by the divergent stretch of 41 amino acids. To further examine the role of this region of the MCAT proteins in cationic amino acid transport, chimeric cDNAs were constructed encoding proteins in which 81 amino acids containing the divergent region of MCAT-2A or MCAT-2B were inserted into MCAT-1 (MCAT-1/2A and MCAT-1/2B), and the equivalent portion of MCAT-1 was inserted into MCAT-2 (MCAT-2/1). In Fig. 4, the deduced amino acid sequences of the exchanged regions are shown, and residues that differ between the three transport proteins are highlighted. The behavior of these chimeric proteins was assessed in Xenopus oocytes. Substitution of the MCAT-2A-spe-
characteristic region increased the apparent $K_m$ of MCAT-1 for arginine from 0.14–0.25 to 1.43–1.57 mM (Table II). Also, the apparent $K_m$ of MCAT-1/2B was 0.45–0.54 mM, slightly greater than MCAT-2B (0.25–0.38), and the $K_m$ of MCAT-2/1, the protein with the reciprocal exchange, was 0.19–0.23 mM, similar to MCAT-1 (0.14–0.25). The maximal velocity of each chimeric protein was also best predicted by the behavior of the transporter that donated this domain (Table II). MCAT-1/2A-mediated efflux of arginine into a salt solution free of cationic amino acids was more than 20-fold greater than MCAT-2/1 and 10-fold greater than MCAT-1/2B (Table III). The increase in arginine efflux upon addition of arginine to the incubation medium was 15-fold for MCAT-2/1, but only 2.5-fold for MCAT-1/2A. MCAT-1/2B-mediated efflux increased 6-fold. A plot of the efflux rates as the percentage of the rate at 0.5 mM demonstrates that the trans-stimulation of MCAT-1/2B was greater than MCAT-2/1, but less than MCAT-1/2A (Fig. 5). When oocytes that expressed MCAT-2/1, MCAT-2B, or MCAT-1/2B were incubated in 10 μM arginine, equilibrium was reached after uptake of 0.8, 0.8, or 2.5 nmol of arginine, respectively (Fig. 6). These findings demonstrate that the intracellular arginine concentration which saturates MCAT-2/1 is one-tenth of MCAT-1/2A and one-third of MCAT-2B. Therefore, both trans-stimulation and outward transport of arginine by these chimeric proteins were best correlated with the behavior of the donor of the exchanged domain and not the backbone.

**DISCUSSION**

In the present study, we have determined that lipopolysaccharide-activated RAW264.7 macrophages express MCAT-2B, a carrier of cationic amino acids that differs from the previously characterized hepatocyte transporter, MCAT-2A, in a single region of 41 amino acid residues. The sequence of MCAT-2B is identical to the corrected sequence of Tea, a protein expressed in murine lymphomas (3). Since the nucleotide sequences of the MCAT-2A and MCAT-2B cDNAs are identical except for the region encoding the divergent domain, it is likely that the MCAT-2A and MCAT-2B mRNAs arise by differential splicing of the same primary transcripts. Although we have not investigated this question directly, we conclude the two proteins are encoded by the same gene. If true, the splicing reaction appears to be regulated in a tissue-specific manner, since MCAT-2A and MCAT-2B are expressed in cells derived from different tissues. Additional studies of the expression of these two proteins are required to examine this question. In macrophages and T cells, MCAT-2B expression requires cell activation. This regulation may reflect a role for MCAT-2B in providing these cells with cationic amino acids as part of the host immune response.

MCAT-2B is the third member of a family of related transporters of cationic amino acids. Like the other MCAT proteins, MCAT-2B has 14 putative membrane-spanning domains and transports substrate in both directions across the plasma membrane. The transport activity is sensitive to trans-stimulation and independent of the presence of extracellular sodium ions. These properties are consistent with the behavior of a carrier protein mediating facilitated diffusion (for review see Ref. 9). The transport properties of MCAT-2B are similar to MCAT-1 and coincide with the $y^+$ phenotype (high affinity and sensitivity to trans-stimulation). Therefore, $y^+$-behavior can be mediated by at least two transporters. It is possible that other well characterized amino acid transport systems may also be comprised of related proteins with tissue-specific expression.

In our previous paper (2), we proposed that the strict dependence of MCAT-1-mediated efflux on the extracellular substrate concentration might be a mechanism to protect cells from depletion of cationic amino acids when the plasma level is low. This hypothesis is supported by the data presented here showing a steep decrease in MCAT-1-mediated efflux from oocytes incubated in medium containing arginine concentrations below the plasma level (0.1 mM). Similarly, MCAT-2B-mediated efflux decreased when the extracellular arginine concentration was below 0.1 mM. However, at very low concentrations of arginine (0 and 0.025 mM), MCAT-2B demonstrated significantly greater activity than MCAT-1. MCAT-2A-mediated efflux was largely independent of the extracellular arginine concentration, confirming our previous results.

Oocytes expressing each transporter accumulated differing steady state levels of arginine when incubated at concentrations that saturate influx. Under these conditions, substrate affinity, and not electrochemical equilibrium, is the sole determinant of the intracellular arginine concentration, assuming the $V_{\text{max}}$ for influx and efflux are equal. Should the $V_{\text{max}}$ for efflux exceed influx, both the affinity and rate of translocation from the inner to the outer face of the membrane determine the intracellular arginine concentration.

The residues in the divergent stretch of 41 amino acids must determine the differences in the transport properties of MCAT-2A and MCAT-2B. Substitution of this region of

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**TABLE II**

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<th>Carrier</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (nmol/oocyte/h)</th>
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<td>1.10–3.40</td>
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**TABLE III**

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<td>4.1±0.56</td>
<td>4.7±0.47</td>
<td>6.5±0.35</td>
</tr>
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</table>
**Characterization of the Third Member of the MCAT Family**

**Fig. 5.** Arginine efflux mediated by the chimeric transporters as a function of the extracellular (trans-) substrate concentration. Oocytes were injected with MCAT-1/2A (squares), MCAT-1/2B (triangles), or MCAT-2/1 (circles) cRNA and two days later assayed as described in the legend to Fig. 2. The data points were calculated as percent of the transport activity observed at 0.5 mM (100%). Each point is the mean from 3 x 3 oocytes ± S.D.

**Fig. 6.** Accumulation of [14C]arginine mediated by the chimeric transporters at an extracellular concentration that saturates influx. Oocytes were injected with MCAT-1/2A (squares), MCAT-1/2B (triangles), or MCAT-2/1 (circles) cRNA and 2 days later assayed as described in the legend to Fig. 3.

MCAT-2A into MCAT-1 was sufficient to confer properties of MCAT-2A; greater $K_m$, increased apparent $V_{max}$, and diminished sensitivity to trans-stimulation by arginine. In addition, the performance of the reciprocal chimeric protein, MCAT-2/1, was most like MCAT-1. This suggests that other differences in the amino acid sequences of MCAT-1 and MCAT-2A are not important in determining the transport properties, even though the two proteins are identical at only 62% of the residues in the optimal alignment. The influence of the substituted domain on the performance of the MCAT-1/2B and MCAT-2/1 proteins was small, but also reflected the properties of MCAT-2B and MCAT-2/1 respectively, which are very similar. Consistent with this observation, the amino acid sequence of the divergent region of MCAT-2B is more closely related to MCAT-1 than to MCAT-2A.

A model for carrier-mediated transport of substrate proposes that MCAT proteins change their conformation during transport, thereby moving the substrate binding site from one side of the membrane to the other (2) (for review see Ref. 9). Accordingly, trans-stimulation can be explained by the dependence of this conformational change on the presence of substrate on both sides of the membrane. Our identification of a region of MCAT that specifies both $K_m$ and dependence on trans-stimulation suggests this region might contain the substrate binding site. However, in studies of another family of carrier proteins, Katagiri and co-workers (10, 11) show that the $K_m$, and $V_{max}$ of glucose transporters can be altered by changes at the C-terminal domain, a region that is distinct from the substrate binding site. Further experiments will be necessary to determine if the divergent domain in MCAT proteins contains the substrate binding site(s), and if true, if this site is translocated in the membrane during transport.

**REFERENCES**